Journal of Chromatography B, 875 (2008) 161–167

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Enantioselective analysis of oxybutynin and *N*-desethyloxybutynin with application to an *in vitro* biotransformation study☆

Patrícia da Fonseca[a], Luis Alexandre Pedro de Freitas[a], Luis Felipe Ribeiro Pinto[b], Cezar Rangel Pestana[a], Pierina Sueli Bonato[a,*]

[a] *Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo (USP), Av. Café SN, CEP 14040-903, Ribeirão Preto, São Paulo, Brazil*
[b] *Departamento de Bioquímica, Instituto de Biologia, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil*

ARTICLE INFO

Article history:
Received 18 February 2008
Accepted 11 May 2008
Available online 17 May 2008

Keywords:
Oxybutynin
N-Desethyloxybutynin
Enantioseparation
Biotransformation

ABSTRACT

An enantioselective method using liquid-phase microextraction (LPME) followed by HPLC analysis was developed for the determination of oxybutynin (OXY) and its major metabolite *N*-desethyloxybutynin (DEO) in rat liver microsomal fraction. The LPME procedure was optimized using multifactorial experiments. Under the optimal extraction conditions, the mean recoveries were 61 and 55% for (*R*)-OXY and (*S*)-OXY, respectively, and 70 and 76% for (*R*)-DEO and (*S*)-DEO, respectively. The validated method was employed to an *in vitro* biotransformation study using rat liver microsomal fraction. The results demonstrated the enantioselective biotransformation of OXY.

1. Introduction

Oxybutynin [4-(diethylamino)-2-butynyl-α-phenylcyclohexane-glycolate hydrochloride] (OXY, Fig. 1) is a chiral drug administrated as a racemic mixture for the treatment of urinary incontinence [1,2]. Anticholinergic agents, such as OXY, inhibit the binding of acetylcholine to the cholinergic receptor and suppress involuntary bladder contractions [3]. After oral administration, OXY is extensively biotransformated by cytochrome P450 enzymes to *N*-desethyloxybutynin (DEO, Fig. 1), the main active metabolite [4]. DEO is present in the plasma at concentrations approximately 4–10 times higher than those of the parent compound and it has similar anticholinergic effects of OXY. In therapeutic use, DEO seems to contribute greatly to the anticholinergic side effects, in particular dry mouth [5,6]. The possibility of OXY delivering via non-oral route may be a more effective method of reducing the drug adverse reactions. Some studies demonstrated that its intravaginal [7] and transdermal [5,6] administration avoid the pre-systemic biotransformation of OXY, minimizing the side effects.

The stereoselectivity in the pharmacokinetic and pharmacodynamic properties of OXY and DEO is demonstrated by differences in the kinetic parameters and in the effects of their enantiomers. Previous studies have reported that (*R*)-OXY and (*R*)-DEO exhibit higher anticholinergic activity than the *S*-enantiomers. In addition, (*S*)-OXY is bound more strongly to glycoprotein than (*R*)-OXY [8], whereas (*R*)-OXY and (*R*)-DEO are eliminated more slowly than the (*S*)-enantiomers [9]. Moreover, based on previous reports that both CYP3A4 and CYP3A5 are involved in the biotransformation of OXY in DEO, it can be suggested that the differences observed in plasma concentration profiles between the enantiomers are caused, at least in part, by the stereoselective biotransformation leaded by these CYP subtypes [9–11].

The enantioselective determination of OXY and DEO in biological samples is described in literature by only three reports. All the authors used chiral columns based on amylose derivates [12] and acid α_1-glycoprotein [5,9]. Most of the methods for the analysis of OXY and DEO enantiomers in biological fluids are based on liquid–liquid extraction (LLE) for sample preparation. However, this technique relies on substantial amounts of organic solvents [13]. In order to overcome this drawback, recent trends in sample pre-treatment techniques have been developed, particularly the miniaturization of LLE by greatly reducing the solvent volume ratio, leading to liquid-phase microextraction (LPME) [14]. In LPME, the aqueous samples are filled in a small vial, and a piece of a porous polypropylene hollow membrane is placed within this sample. LPME could be used in two modes: two or three phases [15]. In the latter system, analytes are extracted from aqueous samples (donor phase) through an organic solvent immobilized in the pores of the hollow membrane (organic phase), and into another

☆ This paper is part of the Special Issue 'Enantioseparations', dedicated to W. Lindner, edited by B. Chankvetadze and E. Francotte.

* Corresponding author. Tel.: +55 16 36024261; fax: +55 16 36024880.
E-mail address: psbonato@fcfrp.usp.br (P.S. Bonato).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.05.023

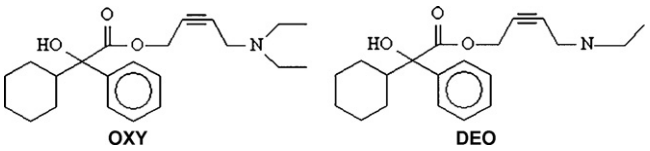

Fig. 1. Oxybutynin and *N*-desethyloxybutynin structures.

aqueous phase (acceptor phase) which is present inside the lumen of the hollow membrane [13,16,17]. In the two-phase system, the acceptor phase is the same organic solvent immobilized inside the membrane.

Many parameters may interfere in the extraction procedure, such as extraction time, temperature, sample agitation, donor phase composition and pH, organic solvent and acceptor phase composition and pH [15]. Therefore, factorial experiments are useful to find the best conditions for the extraction. A factorial experiment is the one whose design consists of two or more factors, each with discrete possible values or "levels", and which the experimental units take on all possible combinations of these levels across all such factors. Such experiment allows studying the effect of each factor on the response variable, as well as, the effects of interactions between factors on the response variable. If the number of experiments for a full factorial design is too high to be logistically feasible, a fractional factorial design may be done, in which some of the possible combinations are omitted [18–20]. A central composite design (CCD) is an experimental design, useful in response surface methodology to build a second-order (quadratic) model for the response variable without requiring a complete three-level factorial experiment. After performing the designed experiment, linear regression is used to obtain the results [18,21].

Based on this, the aim of this study was to develop a LPME extraction procedure followed by chiral HPLC analysis for the enantioselective determination of OXY and DEO in microsomal fraction of rat liver. During method optimization, several parameters were investigated using factorial experiments. Finally, the optimized and validated method was applied to the study of OXY *in vitro* biotransformation using microsomal fraction isolated from rat liver.

2. Experimental

2.1. Chemicals and reagents

Rac-oxybutynin and *rac*-*N*-desethyloxybutynin were generously supplied by Watson Laboratories (Corona, CA, USA) and Orgamol (Switzerland, France), respectively. The solvents used for mobile phase preparation, ethanol from Merck (Darmstadt, Germany), hexane from Mallinckrodt (Phillipsburg, NJ, USA) and 2-propanol from EM Science (Gibbstown, NJ, USA) were all HPLC grade. Diethylamine, analytical grade, was obtained from Fluka (Buchs, Switzerland). Di-*n*-hexyl ether and 1-octanol were purchased from Alfa Aesar (Ward Hill, MA, USA) and Sigma (St. Louis, MO, USA), respectively. Sodium hydroxide and perchloric acid were from Nuclear (São Paulo, SP, Brazil). Sodium dihydrogen phosphate and sodium hydrogen phosphate were obtained from Merck (Darmstadt, Germany). β-Nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Sigma (St. Louis, MO, USA). Purified water was obtained from a Milli-Q-System (Millipore, Mildford, MA, USA).

2.2. Apparatus and chromatographic conditions

The chromatographic analyses were conducted using a Shimadzu (Kyoto, Japan) liquid chromatograph, equipped with an LC-AT VP solvent pump unit and an SPD-10A UV–vis detector operating at 262 nm. Injections were performed manually through a 50 μL loop with a model 7125 Rheodyne injector (Rheodyne, Cotati, CA, USA). Data were collected using a CR6A integrator (Shimadzu, Kyoto, Japan).

The resolution of OXY and DEO enantiomers were evaluated at 22 °C using a Chiralpak AD column (250 mm × 4.6 mm, 10 μm particle size, Chiral Technologies, Exton, PA, USA). A NH_2 LiChroCart column (4 mm × 4 mm, 5 μm particle size, Merck, Darmstadt, Germany) was used as guard column. The best resolution was achieved using hexane–isopropanol–ethanol (95:4:1, v/v/v) plus 0.3% diethylamine as the mobile phase, at a flow rate of 0.9 mL min^{-1}.

2.3. Standard solutions and quality control samples

OXY and DEO stock (1 mg mL^{-1}) and working solutions (25–400 and 20–400 μg mL^{-1}, respectively) were prepared in methanol, stored frozen at −20 °C and protected from light, remaining stable for at least 3 months.

Drug-free human plasma samples were obtained from healthy volunteers and stored frozen at −20 °C. Prior to use, the diluted plasma samples were allowed to thaw at room temperature. Diluted plasma samples spiked with 400, 1250 and 3500 ng mL^{-1} of each OXY enantiomer and 325, 1000 and 3500 ng mL^{-1} of each DEO enantiomer were prepared to measure the accuracy and precision of the method (quality control samples).

2.4. Optimization of the liquid-phase microextraction procedure

To minimize the use of animals, the development and validation of the method was performed using drug-free plasma obtained from healthy human volunteers instead of microsomal fraction from rat liver homogenate. Differences in extraction behavior between plasma and microsomal fraction were not expected because the amount of plasma and microsomal fraction used were very small (less than 1% of the total sample) and the protein concentration in the sample was similar using plasma or microsomal fraction. The plasma was diluted with 1 mol L^{-1} sodium phosphate buffer, pH 7.4 (17 μL plasma/983 μL buffer) in order to achieve protein concentrations similar to those of the microsomal incubation mixtures [22].

Diluted plasma samples (1000 μL) spiked with OXY and DEO solutions were transferred to amber tubes followed by the addition of 400 μL perchloric acid 6% (v/v). The buffer and perchloric acid solutions were added to simulate the conditions used in the incubation experiments, i.e., the pH adjustment to assure enzymatic reaction and the perchloric acid addition to stop the incubation reaction. Finally, 200 μL sodium hydroxide 2 mol L^{-1} was also added to alkalinize the samples to pH 8.0, required for the extraction of the basic analytes. Next, the volume was adjusted to 4.0 mL with 1 mol L^{-1} sodium phosphate buffer, pH 8.0. For each extraction, a new 6 cm length polypropylene hollow membrane was placed in the system, and 1-octanol and di-*n*-hexyl ether were evaluated as organic solvents immobilized in the pores. The excess of solvent in the membrane was removed by 10 s of stirring in water. Three acid solutions were evaluated as acceptor phase: perchloric acid 0.1 mol L^{-1}, acetic acid 0.1 mol L^{-1} and trifluoracetic acid 0.1 mol L^{-1}.

Factorial experiments were used to optimize other parameters that can interfere in the LPME extraction. A fractional factorial design was first developed (Tables 1 and 2) with six factors and two

Table 1
Factors and their levels in the fractional factorial design

Factor	Level	
	Low	High
Extraction time (min)	20 (A)	40 (a)
Acid concentration in the acceptor phase (mol L^{-1})	0.05 (B)	0.2 (b)
Methanol (%, v/v)	0 (C)	5 (c)
Agitation (rpm)	1500 (D)	4500 (d)
NaCl (%, w/v)	0 (E)	20 (e)
Donor phase pH	7 (F)	8 (f)

rpm: rotation per minutes.

Table 2
Combinations studied in the fractional factorial design

Factor level	Combination							
	1	2	3	4	5	6	7	8
A or a	A	A	A	A	a	a	a	a
B or b	B	b	B	b	B	b	B	b
C or c	C	C	c	c	c	c	C	C
D or d	D	d	D	d	d	D	d	D
E or e	E	e	e	E	E	e	e	E
F or f	F	f	f	F	f	F	F	F
Results	s	t	u	v	w	x	y	z

levels: donor phase pH, acid concentration in the acceptor phase, extraction time, methanol percentage, sample agitation and salt concentration. The response evaluated in these experiments was the sum of the peaks areas of OXY and DEO enantiomers.

In this study the influence of each factor in the OXY and DEO extraction was analyzed by the difference between the means of the analyte chromatographic areas in the studies using low and high levels; the factor was considered important in the extraction procedure when this difference was a high number. The equation bellow describes how the influence of each factor was calculated:

$$\text{factor} = |\text{mean of OXY or DEO areas of low levels}| - |\text{mean of OXY or DEO areas of high levels}|$$

According to the factorial fraction design results, a new study using a central composite design (CCD) with four factors and five levels was developed (Table 3). This study was outlined with 27 experiments (Table 4). Since OXY and DEO had similar extraction characteristics, only the sum of the peaks areas of OXY enantiomers was used to evaluate the results obtained in CCD experiments. The "General Linear Model" software "Statistica 99 Edition—Kernel release 5.5" (Statsoft Inc., Tulsa, OK, USA) was used to evaluate the CCD results.

2.5. Method validation

Calibration curves were constructed by analyzing diluted human plasma samples (1000 μL, $n = 3$ for each concentration) spiked with 25 μL of OXY and DEO standard solutions, to obtain concentration ranges of 312–5000 ng mL^{-1} for each OXY enantiomer and 250–5000 ng mL^{-1} for each DEO enantiomer. Plots of peak height *versus* OXY and DEO concentrations were constructed and the linear regression lines were used for determination of the enantiomers concentration in the samples. The linearity of the analytical method was determined in a similar way.

Table 3
Factors and their levels in the central composite design

Levels	Time (min) (X_1)	% Methanol (X_2)	Agitation (rpm) (X_3)	% NaCl (X_4)
$-\alpha$	14.5	1.87	1500	2.25
−1	20	2.98	2000	5.00
0	30	5.00	3000	10.00
1	40	7.02	4000	15.00
α	45.5	8.13	4500	17.75

rpm: rotation per minutes.

Table 4
Combinations studied in the central composite design

Experimental numbers	X_1	X_2	X_3	X_4
1	−1	−1	−1	−1
2	1	−1	−1	−1
3	−1	1	−1	−1
4	1	1	−1	−1
5	−1	−1	1	−1
6	1	−1	1	−1
7	−1	1	1	−1
8	1	1	1	−1
9	−1	−1	−1	1
10	1	−1	−1	1
11	−1	1	−1	1
12	1	1	−1	1
13	−1	−1	1	1
14	1	−1	1	1
15	−1	1	1	1
16	1	1	1	1
17	0	0	0	0
18	0	0	0	0
19	0	0	0	0
20	$-\alpha$	0	0	0
21	α	0	0	0
22	0	$-\alpha$	0	0
23	0	α	0	0
24	0	0	$-\alpha$	0
25	0	0	α	0
26	0	0	0	$-\alpha$
27	0	0	0	α

The DEO quantification limit was assayed by analyzing aliquots of diluted human plasma ($n = 5$) spiked at concentrations of 250 ng mL^{-1} of each enantiomer against a calibration curve in the concentration range of 250–5000 ng mL^{-1}.

The precision and accuracy of the method were evaluated by within-day ($n = 5$) and between-day ($n = 3$) assays using spiked diluted plasma with OXY and DEO at concentrations of 400, 1250 and 3500 ng mL^{-1} of each OXY enantiomer and 325, 1000 and 3500 ng mL^{-1} of each DEO enantiomer.

The recovery of OXY and DEO enantiomers extracted from diluted plasma samples ($n = 3$) spiked with 400, 1250 and 3500 ng mL^{-1} of each OXY enantiomer and 325, 1000 and 3500 ng mL^{-1} of each DEO enantiomer was determined using calibration curves obtained by the direct analysis of the analytes in the mobile phase.

Freeze-thaw cycle and short-term room temperature stability assays were also evaluated. To perform the freeze-thaw cycle stability assay, three aliquots at low (400 and 325 ng mL^{-1} for OXY and DEO enantiomers, respectively) and high (3500 ng mL^{-1}) concentrations of the control samples (prepared in microsomal fraction) were stored at −20 °C for 24 h and thawed at room temperature. When completely thawed, the samples were refrozen for 12 h under the same conditions. This cycle was repeated twice more. Finally, the samples were extracted and analyzed. For the determination of short-term room temperature stability assay, three aliquots of samples (at the same concentrations as described above) were prepared and kept at room temperature (23 °C) for 12 h. After this period the samples were analyzed. The peak height ratio obtained from both stability assays were compared to the peak height ratio obtained with freshly prepared samples. Student's *t*-test was applied, with the level of significance set at $p < 0.05$ [23].

2.6. In vitro biotransformation study

The microsomal fraction was obtained from male Wistar rat (180–200 g) liver homogenates by ultracentrifugation procedures [24] and it was stored frozen at −70 °C. The incubation system consisted of 0.4 mg mL^{-1} of protein, racemic OXY (10 μg mL^{-1}), 716 μL sodium phosphate buffer 1 mol L^{-1} (pH 7.4) and 250 μL NADPH-regenerating system (NRS) (1.7 mg mL^{-1} NADP, 7.8 mg mL^{-1} glucose-6-phosphate and 1.5 units mL^{-1} glucose-6-phosphate dehydrogenase in Tris–HCl buffer (50 mmol L^{-1}/KCl 0.154 mol L^{-1}, pH 7,4). The incubation was carried out in a 37 °C shaking water bath for 3 min. The incubation was stopped by adding 400 μL of perchloric acid 6% and then the samples were submitted to extraction and chromatographic analysis. Product formation was linear under the conditions described above.

3. Result and discussion

3.1. Chromatographic resolution of OXY and DEO enantiomers

Initially, the chiral resolution of OXY and DEO was developed using a chiralpak AD column and hexane: isopropanol (9:1, v/v) as mobile phase at a flow-rate of 1.0 mL min^{-1}, but in this condition the separation was not satisfactory. After changing several parameters, the separation of OXY and DEO enantiomers through this column was possible using hexane: isopropanol: ethanol (95:4:1, v/v/v) plus 0.3% DEA as mobile phase. Under this condition, we succeeded a separation in 20 min (Fig. 2B).

The elution order was determined by the analysis of individual enantiomers (previously separated and collected according to the proposed method) under the chromatographic conditions established by Alebic-Kolbach and Zavitsanos [12]. The first OXY and DEO enantiomer peaks analyzed correspond to (*R*)-enantiomers and the last ones correspond to (*S*)-enantiomers.

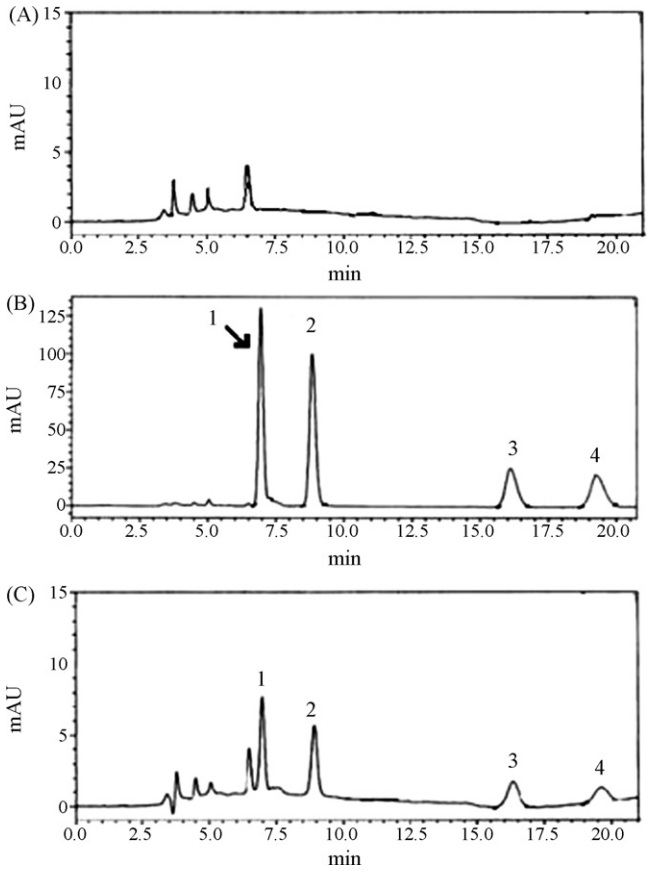

Fig. 2. Chromatograms referring to the analysis of (A) drug-free microsome preparation obtained from rat liver homogenates, (B) microsome preparation spiked with OXY and DEO enantiomers and (C) microsome preparation after incubation procedure: (*R*)-OXY (1) and (*S*)-OXY (2); (*R*)-DEO (3) and (*S*)-DEO (4). Chromatographic conditions as in Section 2.2. Incubation condition: 3 min of incubation, 0.4 mg mL^{-1} of protein and 5000 ng mL^{-1} of each OXY enantiomer in incubation system.

3.2. Optimization of the LPME procedure

In LPME the analytes are transferred from large sample volume (1–5 mL) to micro-sample volume (5–50 μL), so this technique allows high preconcentration of the analytes; in addition, excellent cleanup has been observed even from biological samples [13]. Among the several factors that potentially affect LPME extraction, the most important were investigated here: organic solvent type, acceptor phase type, time extraction, stirring rate, sample pH, salt and methanol addition to donor phase.

In preliminary experiments, we established the organic solvent and the acceptor phase. The organic solvent used in LPME must be compatible with the membrane, immiscible in water as well as it must have low volatility [25]. Two solvents with these characteristics were evaluated, i.e., 1-octanol and di-*n*-hexyl ether. The last one resulted in higher recoveries for OXY and DEO enantiomers, so it was selected for subsequent experiments. For extraction of basic compounds the maximal trapping of extracted analyte in ionized form requires low pH value of the acceptor phase [26]. Three acid solutions were selected to be studied: perchloric acid 0.1 mol L^{-1}, acetic acid 0.1 mol L^{-1} and trifluoracetic acid 0.1 mol L^{-1}. The highest analyte recoveries were obtained using trifluoracetic acid 0.1 mol L^{-1}.

After these experiments, a fractional factorial design was used and the results obtained are demonstrated in Table 5. The factor that demonstrated the lowest influence in the OXY and DEO extraction was the concentration of the acid in the acceptor phase, so trifluoracetic acid 0.1 mol L^{-1} was selected for subsequent experiments and this factor was not evaluated in the CCD. The donor phase pH exerted great influence in the extraction of the analytes, but it was not evaluated in the CCD because OXY has stability problems in pH values higher than 8.0 [3]. So, the CCD study was outlined with four factors: extraction time, sample agitation and salt and methanol addition to donor phase.

The microsomal fraction proteins can interact with drugs and make difficult their extraction. So, the addition of organic solvents to the samples, such as methanol, could be used to suppress these interactions increasing the analytes recoveries. The effect of methanol and NaCl in the donor phase is shown in Fig. 3; the response surface shows that methanol content did not affect the OXY peak area for all NaCl concentrations in the range studied. The effect of NaCl content is clearly more pronounced and the surface curvature shows a quadratic effect. The increase in NaCl content caused a decrease in OXY peak area, which seems to be more pro-

Table 5
Evaluation of each factor studied in fractional factorial design

Factor	Differences between the higher and lower levels	
	OXY	DEO
Time	−15084	−7574
Acceptor phase	−1865	−1067
Methanol	−7994	−6127
Agitation	−3772	2460
NaCl	6906	33824
Donor phase pH	10780	−6712

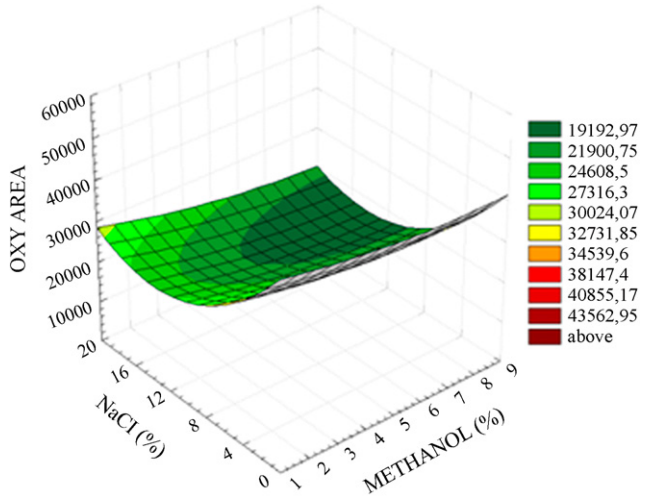

Fig. 3. Response surface (methanol and NaCl percentage) obtained by CCD where the response was total OXY chromatographic peak area.

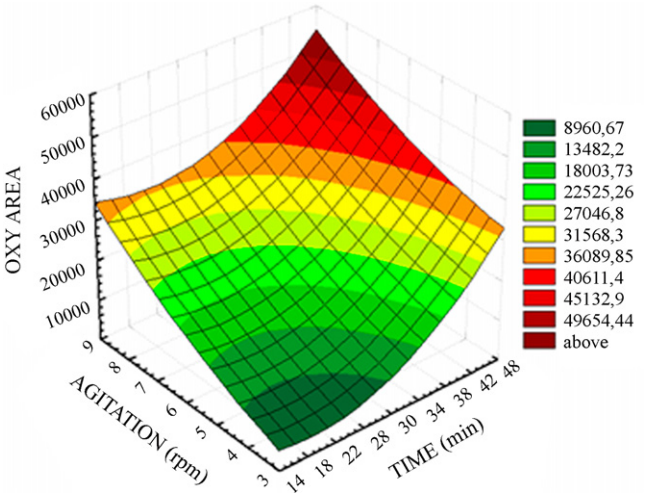

Fig. 4. Response surface (agitation and time extraction) obtained by CCD where the response was total OXY chromatographic peak area.

nounced for lower concentrations and an asymptotic tendency for high NaCl concentrations. The results observed in Fig. 3 were confirmed by the analysis of variance (ANOVA) using response surface methodology, as shown in Table 6. Neither linear nor quadratic terms of methanol were significant, although there is an interaction with extraction time with lower OXY peak area in longer extraction time, which is possibly related to methanol evaporation during extraction. However, the linear term of NaCl concentration is significant at 1% level, which attests a strong negative effect of this factor for OXY extraction. The effect of time and agitation are shown in Fig. 4; the response surface shows the great influence of those factors on OXY extraction. In this figure, OXY peak area increased strongly with both factors, reaching a maximum for the highest agitation speed and longer extraction time. The effect of agitation speed seems to be linear while there is a remarkable curvature in the extraction time effect. Those results were confirmed by the analysis of variance showed in Table 6. The linear terms of time and agitation were both significant at 0.1%, while the quadratic term of time was significant at 10% level. As mentioned before, time also showed an interaction with methanol content. The effects of those factors on OXY extraction could be reliably evaluated by analysis of variance on the experimental data, as shown in Table 6, since many factors presented high significance levels.

The ANOVA showed that the linear effects of time, agitation and NaCl concentration, the quadratic effect of time and the interaction between time × methanol were significant at 10% level. In this work, only factors with significance levels higher than 10% were considered. The fitted equation, with correlation coefficient $r^2 = 0.9145$, is given by

$$\mathrm{OXY} = 20220.99 + 7419.93\left(\frac{T-30}{10}\right) + 3766.03\left(\frac{T-30}{10}\right)^2 + 8341.66\left(\frac{A-6}{2}\right) - 4858.95\left(\frac{N-10}{5}\right) - 3221.16\left(\frac{T-30}{10}\right)\left(\frac{M-5}{2.02}\right)$$

where T: time, A: agitation, N: NaCl concentration and M: methanol percentage.

In this study it was not possible to find the optimum values for extraction by canonical analysis, but the greatest OXY and DEO recoveries were obtained with highest extraction time and agitation speed and lowest methanol and NaCl concentration in samples. A new study was not outlined due to equipment limitation (agitation at 4500 rpm is the highest level of the equipment) and time limitations (long extraction time turns the analyses impracticable).

Table 6
Analysis of variance on LPME data

Factor	Sum of square	Degree of freedom	Mean square	F_{calc}	p
T	$115{,}443 \times 10^4$	1	$115{,}443 \times 10^4$	32.5278	0.0001*
M	$581{,}441 \times 10^2$	1	$581{,}441 \times 10^2$	1.6512	0.2230
A	$144{,}768 \times 10^4$	1	$144{,}768 \times 10^4$	41.1111	0.00003*
N	$491{,}194 \times 10^3$	1	$491{,}194 \times 10^3$	13.9490	0.0028**
T^2	$164{,}353 \times 10^3$	1	$164{,}353 \times 10^3$	4.6673	0.0517***
M^2	2,118,332	1	2,118,332	0.0602	0.8104
A^2	$137{,}678 \times 10^2$	1	$137{,}678 \times 10^2$	0.3910	0.5435
N^2	$104{,}067 \times 10^3$	1	$104{,}067 \times 10^3$	2.9553	0.1113
T × M	$166{,}014 \times 10^3$	1	$166{,}014 \times 10^3$	4.7144	0.0507***
T × A	$110{,}864 \times 10^2$	1	$110{,}864 \times 10^2$	0.3148	0.5851
T × N	$444{,}339 \times 10^2$	1	$444{,}339 \times 10^2$	1.2618	0.2833
M × A	$110{,}481 \times 10^2$	1	$110{,}481 \times 10^2$	0.3137	0.5857
M × N	756,683	1	756,683	0.0215	0.8859
A × N	84,754	1	84,754	0.0024	0.9617
Error	$422{,}566 \times 10^3$	12	$352{,}138 \times 10^2$		
Total	$408{,}136 \times 10^4$	26			

T: time, M: methanol, A: agitation, N: NaCl, *0.1%, **1% and ***10%.

Table 7
Precision and accuracy for the analysis of OXY enantiomers

	Within-day ($n = 5$)			Between-day ($n = 3$)		
	Concentration (ng mL^{-1})	CV (%)	E (%)	Concentration (ng mL^{-1})	CV (%)	E (%)
400 (ng mL^{-1})						
(*R*)-OXY	454	9.3	13.4	452	12.5	12.9
(*S*)-OXY	448	10.0	12.0	458	7.8	14.6
1250 (ng mL^{-1})						
(*R*)-OXY	1380	5.2	10.5	1414	9.7	13.1
(*S*)-OXY	1323	5.5	5.9	1382	6.6	10.6
3500 (ng mL^{-1})						
(*R*)-OXY	3995	4.3	14.1	3684	11.2	5.3
(*S*)-OXY	4024	4.9	14.8	3982	10.2	13.8

n: number of samples; CV: coefficient of variation; *E*: relative error.

Based on the experiments discussed above, the best LPME condition was established as no salt and methanol in donor phase, sample agitation at 4500 rpm, 45 min of extraction, trifluoracetic acid 0.1 mol L^{-1} as acceptor phase, di-*n*-hexyl ether as the extraction solvent and donor phase pH adjustment to 8.0. Under these conditions excellent cleanup of the samples was obtained as shown in Fig. 2A.

The LPME optimization using multivariable design allowed a reduction in the amount of required experiments, making it possible to distinguish interactions among the factors that would not be detectable by classical optimization procedure.

3.3. Method validation

In order to evaluate the practical applicability of the LPME technique, optimal extraction conditions were validated analyzing some important parameters, such as linearity, recovery, precision, accuracy, quantification limit and stability. Coefficients of variation and relative errors of less than 15% were considered acceptable, except for the quantification limit, for which these values were established at 20%, as recommended in the literature [27–29].

During method validation, the area and height of OXY and DEO enantiomers peaks were used. The height of the peaks was chosen for the calculations because of better reproducibility.

The method proved to be linear over the concentration range of 312–5000 ng mL^{-1} of each OXY enantiomer and 250–5000 ng mL^{-1} of each DEO enantiomer, with typical calibration curve equations determined as $y = 1.5727x - 87.263$ and $y = 1.2082x + 245.25$ for (*R*)-OXY and (*S*)-OXY, respectively, and $y = 0.8764x - 51.496$ and $y = 0.728x + 67.788$ for (*R*)-DEO and (*S*)-DEO, respectively. The correlation coefficients obtained ($r^2 \geq 0.98$) show that the method attends the criteria for methods used in the analysis of drugs in biological samples [27–29]. In addition, the coefficients of variation on the slopes and intercepts were lower than 15%.

The LPME recoveries were 61 and 55% for (*R*)-OXY and (*S*)-OXY, respectively, and 70 and 76% for (*R*)-DEO and (*S*)-DEO, respectively. The differences between recoveries of the enantiomers of OXY and DEO are within the experimental errors expected for this procedure (≤15%). Comparing the literature information on microextraction techniques [30], these recoveries were considered very good, especially taking into account the limitations of pH (≤8.0) because of the stability of OXY. The quantification limit determined from spiked plasma was 250 ng mL^{-1} for each DEO enantiomer and

Table 8
Precision and accuracy for the analysis of DEO enantiomers

	Within-day ($n = 5$)			Between-day ($n = 3$)		
	Concentration (ng mL^{-1})	CV (%)	E (%)	Concentration (ng mL^{-1})	CV (%)	E (%)
325 (ng mL^{-1})						
(*R*)-DEO	293	14.8	−9.8	295	13.6	−9.1
(*S*)-DEO	300	14.7	−7.6	307	14.8	−5.5
1000 (ng mL^{-1})						
(*R*)-DEO	944	10.3	−5.5	944	14.1	−5.6
(*S*)-DEO	970	9.8	−2.9	980	9.5	−1.9
3500 (ng mL^{-1})						
(*R*)-DEO	4021	6.2	14.9	3760	14.2	7.4
(*S*)-DEO	3922	7.7	12.0	3950	6.8	12.9

n: number of samples; CV: coefficient of variation; *E*: relative error.

Table 9
Stability test for the analysis of OXY and DEO enantiomers in microsomal fraction

Nominal concentration (ng mL^{-1})	*p*-Values		Nominal concentration (ng mL^{-1})	*p*-Values	
	(*R*)-OXY	(*S*)-OXY		(*R*)-DEO	(*S*)-DEO
Freeze-thaw cycles					
400	0.9711	0.2847	325	0.7274	0.9562
3500	0.8553	0.1063	3500	0.6037	0.4340
Short-term room temperature					
400	0.8577	0.1621	325	0.0924	0.1275
3500	0.2842	0.6610	3500	0.9284	0.9910

this limit was considered to be the lowest concentration quantified with a relative error lower than 20%. The quantification limit was not determined for OXY due to the high concentration of the drug used in the biotransformation experiments. The three methods described in literature for the analysis of DEO enantiomers use liquid chromatography/tandem mass spectrometry (LC–MS–MS). Because LC–MS–MS is a very sensitive technique, these authors reached low values of limit of quantification (approximately 0.5 ng mL^{-1} for each OXY and DEO enantiomer) [5]. In our study, we find higher limits of quantification for DEO enantiomers; however, these values were sufficient to allow the application of this method in an *in vitro* biotransformation study.

The analytical precision of the method was expressed as within-day and between-day CV (%) whereas the accuracy was expressed as relative error (%). The results are summarized in Tables 7 and 8. The stability of OXY and DEO at pH 8.0 was assured in these experiments, because the results obtained for samples prepared and immediately extracted were not significantly different than those obtained for sample prepared and extracted up to 6 h.

The stability study showed no statistical difference between freeze-thaw cycle and short-term room temperature stability assays with p-values > 0.05 (Table 9).

3.4. Method application

The validated method was applied to an *in vitro* biotransformation study using rat liver microsomal fraction. Fig. 2C shows the chromatogram obtained by the analysis of OXY submitted to the biotransformation procedure. The protein concentration, time of incubation and substrate concentration were optimized in order to evaluate the linear OXY biotransformation and the established conditions were 3 min of incubation, 0.4 mg mL^{-1} of protein and 5000 ng mL^{-1} of each OXY enantiomer in the incubation system. Under this incubation condition K_m of 9.3 and 7.9 nmol L^{-1} for (*R*)-OXY and (*S*)-OXY, respectively, were observed indicating greater enzyme affinity to (*S*)-OXY and consequently, the enantioselective OXY biotransformation by rat liver microsomes. Previous studies have clearly demonstrated the enantioselective pharmacokinetic characteristics of OXY and DEO after oral or transdermal administration in man [5,6], however only one recent report described the *in vitro* stereoselective biotransformation of OXY using human microsomal fraction [9] as well as recombinant human P450-expressing microsomes. Comparing our preliminary results with those reported by Mizushima et al. [9], the *R*/*S* ratio K_m of OXY is similar in human and rat microsomes.

4. Conclusion

A three phase LPME method for the enantioselective analysis of OXY and DEO in microsomal preparations was described. LPME proved to be a powerful technique for sample preparation, providing high recoveries, efficient cleanup and low consume of organic solvent. The use of experimental design in LPME optimization was very useful, since it reduced the number of experiments and it made possible the study of the interactions between factors. Finally, the validated method has a proven viability for quantitative analysis of OXY and DEO enantiomers in liver microsomal fraction.

Acknowledgements

The authors are grateful to Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support and for granting research fellowships. In addition, the authors wish to express their thanks to Prof. Dr. Carlos Curti for the use of his laboratory facilities during microsomal fraction isolation.

References

[1] H. Kim, S.B. Han, J. Pharm. Biomed. Anal. 31 (2003) 341.
[2] E. Miyamoto, Y. Demizu, Y. Murata, Y. Yamada, S. Kawashima, J. Chromatogr. A 653 (1993) 135.
[3] R. Massoud, G. Federici, S. Casciani, S.M. Di Stasi, G. Fucci, A. Giannantoni, C. Cortese, J. Chromatogr. B 734 (1999) 163.
[4] E. Lukkari, P. Taavitsainen, A. Juhakoski, O. Pelkonen, Pharmacol. Toxicol. 82 (1998) 161.
[5] R.H. Zobrist, B. Schmid, A. Feick, D. Quan, S.W. Sanders, Pharm. Res. 18 (2001) 1029.
[6] R.H. Zobrist, D. Quan, H.M. Thomas, S. Stanworth, S.W. Sanders, Pharm. Res. 20 (2003) 103.
[7] A.D. Woolfson, R.K. Malcolm, R.J. Gallagher, J. Control. Release 91 (2003) 465.
[8] A. Shibukawa, N. Ishizawa, T. Kimura, Y. Sakamoto, K. Ogita, Y. Matsuo, Y. Kuroda, C. Matayatsuk, T. Nakagawa, I.W. Wainer, J. Chromatogr. B 768 (2002) 177.
[9] H. Mizushima, K. Takanaka, K. Abe, I. Fukazawa, H. Ishizuka, Xenobiotica 37 (2007) 59.
[10] E. Lukkari, P. Taavitsainen, A. Juhakoski, O. Pelkonen, Pharmacol. Toxicol. 84 (1998) 161.
[11] M. Yaich, M. Popon, Y. Medard, E.J. Aigrain, Pharmacogenetics 8 (1998) 449.
[12] T. Alebic-Kolbach, A.P. Zavitsanos, J. Chromatogr. A 759 (1997) 65.
[13] S. Pedersen-Bjergaard, K.E. Rasmussen, J. Chromatogr. B 817 (2005) 3.
[14] T.S. Ho, J.L.E. Reubsaet, H.S. Anthonsen, S. Pedersen-Bjergaard, K.E. Rasmussen, J. Chromatogr. A 1072 (2005) 29.
[15] E. Psillakis, N. Kalogerakis, Trends Anal. Chem. 22 (2003) 565.
[16] K.E. Rasmussen, S. Pedersen-Bjergaard, M. Krogh, H.G. Ugland, T. Gronhaug, J. Chromatogr. A 873 (2000) 3.
[17] K.E. Rasmussen, S. Pedersen-Bjergaard, Trends Anal. Chem. 23 (2004) 1.
[18] S.L.C. Ferreira, R.E. Bruns, E.G.P. Silva, W.N.L. Santos, C.M. Quintella, J.M. David, J.B. Andrade, M.C. Breitkreitz, I.C.S.F. Jardim, B.B. Neto, J. Chromatogr. A 1158 (2007) 2.
[19] B.B. Neto, I.S. Scarminio, R.E. Bruns, Quim. Nova 29 (2006) 1401.
[20] M. Mousavi, E. Noroozian, M. Jalali-Heravi, A. Mollahosseini, Anal. Chim. Acta 581 (2007) 71.
[21] E.T. Sousa, F.M. Rodrigues, C.C. Martins, F.S. Oliveira, P.A.P. Pereira, J.B. Andrade, Microchem. J. 82 (2006) 142.
[22] J. Ducharme, R. Farinotti, J. Chromatogr. B 698 (1997) 243.
[23] H. Rosing, W.Y. Man, E. Doyle, A. Bult, J.H. Beijnen, J. Liq. Chromatogr. Relat. Technol. 23 (2000) 329.
[24] B.G. Lake, in: K. Snell, B. Mullock (Eds.), Biochemical Toxicology—A Practical Approach, IRL Press, Oxford, Washington, DC, 1987, p. 183.
[25] G. Shen, H.K. Lee, Anal. Chem. 74 (2002) 648.
[26] H.G. Ugland, M. Krogh, L. Reubsaet, J. Chromatogr. B 798 (2003) 127.
[27] FDA, Guidance for Industry: Bioanalytical Methods Validation, May 2001.
[28] F. Bresolle, M. Bromet-Petit, M. Audran, J. Chromatogr. B 686 (1996) 3.
[29] R. Causon, J. Chromatogr. B 689 (1997) 175.
[30] H. Lord, J. Pawliszyn, J. Chromatogr. A 902 (2000) 17.