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Short communication

Determination of pindolol enantiomers in amniotic fluid and breast milk by high-performance liquid chromatography: Applications to pharmacokinetics in pregnant and lactating women

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Abstract

A method for the determination of pindolol enantiomers in amniotic fluid and breast milk was developed, validated, and applied to the investigation of six pregnant women treated with rac-pindolol (10 mg/12 h). Biological samples were extracted with *tert*-methyl-butyl ether, and the pindolol enantiomers were resolved on a Chiralpak AD column. Amniotic fluid/plasma and milk/plasma concentrations ratios ranged from 0.4 to 4.5 and from 0.6 to 3.7, respectively, for (+)-*R*-pindolol and from 0.5 to 3.5 and from 1.1 to 2.8, respectively, for (−)-*S*-pindolol. Preliminary data suggest that amniotic fluid and breast milk are routes of fetal exposure to pindolol enantiomers.

Keywords: Pindolol; Enantiomers; Amniotic fluid; Breast milk; Pharmacokinetics; Pregnancy

1. Introduction

Pindolol is an effective agent for treating hypertension in pregnancy, a disease that complicates up to 5% of all pregnancies [1–4]. Pindolol causes no changes in utero or in umbilicoplacental vascular impedance or blood flow, has no effect on fetal haemodynamics, and does not affect fetal cardiac function. [2]. It is marketed as a racemic mixture of the (−)-*S* and (+)-*R* enantiomers. Because pindolol is eliminated stereoselectively by the kidney [5] and the pharmacological activity of (−)-*S*-pindolol is considerably higher than that of the (+)-*R* enantiomer [6,7], it is important to evaluate each enantiomer individually in biological fluids after racemic pindolol administration.

The pharmacokinetics and transplacental distribution of pindolol enantiomers at delivery in pregnancy-induced hypertension were reported only in a study by Gonçalves et al. [8]. The kinetic disposition of pindolol in maternal plasma was stereoselective with lower total clearance values (Cl/f 62.5 L/h versus 55.7 L/h) and higher renal clearance values (9.2 L/h versus 10.9 L/h) for (−)-*S*-pindolol. Pindolol crosses the placenta, with cord to maternal serum ratios at 2 and 6 h after the last dose equal to 0.4 and 0.7, respectively [1]. The transplacental distribution of pindolol was reported not to be stereoselective [8].

There are no data available concerning the distribution of pindolol as an enantiomeric mixture or as individual enantiomers in amniotic fluid. Despite increasing interest in defining drug transfer between mother and fetus, some pathways of fetal exposure have been incompletely investigated. One route of fetal exposure for which sparse information exists is amniotic fluid. In later pregnancy, fetal urine is the principal constituent of amniotic fluid [9]. The fetus swallows 210–760 mL/day of amniotic fluid by term. If amniotic fluid contains significant pindolol concentrations, it may represent a medium for continuous fetal exposure to the maternally administered β-blocker [9].

Although the manufacturer states that pindolol is secreted in human milk, no published reports are available describing pindolol levels in milk [2]. The drug level in milk depends on

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the physiochemical properties of the drug, the degree of plasma protein binding, and the maternal serum concentration. Pindolol is a weak base with pK_a equal to 9.7. It is moderately lipid-soluble [10] and about 40–60% is reported to be bound to plasma proteins [11].

No methods have been reported for the analysis of pindolol as an enantiomeric mixture or as individual enantiomers in amniotic fluid or breast milk. The enantioselective determination of pindolol in plasma and urine has been performed by high-performance liquid chromatography (HPLC) with fluorescence or ultraviolet detection or liquid chromatography/tandem mass spectrometry (LC–MS–MS).

HPLC methods for plasma or urine determination have been reported after direct (chiral columns) or indirect (chiral derivatization) enantioseparation. Indirect methods for the analysis of pindolol enantiomers employ (−)-*S*-α-methylbenzyl isocyanate [5,6,12,13] or 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate [14] for the formation of pindolol diastereoisomers which are separated on a reverse-phase C-18 column, with quantitation limits of 2.0–10.0 ng/mL plasma for both enantiomers. Direct methods use chiral columns consisting of α_1-acid glycoprotein [15], cellulose tris(3,5-dimethylphenyl carbamate) (Chiralcel ODR) [7], or amylose tris-(3,5-dimethylphenylcarbamate) (Chiralpak AD) [8], with detection limits of 1.2–0.5 ng/mL of (+)-*R*-pindolol and 4.3–0.5 ng/mL of (−)-*S*-pindolol in serum, and 21.0–5.0 ng/mL of (+)-*R*-pindolol and 76.0–5.0 ng/mL of (−)-*S*-pindolol in urine.

The enantioselective determination of pindolol in human serum or urine has also been achieved by LC–MS–MS after enantiomer resolution on a phenylcarbamate-β-cyclodextrin column [16] or on a Chirobiotic T column [17]. The authors reported lower limits of quantification, in general of the order of 0.3 ng for each enantiomer/mL plasma or urine.

The present study reports for the first time the development and validation of pindolol enantiomer analysis in amniotic fluid and breast milk using HPLC with fluorescence detection after enantiomers resolution on a Chiralpak AD column. The method was applied to investigate six patients with pregnancy-induced hypertension treated with racemic pindolol (10 mg/12 h). Data concerning maternal plasma and urine disposition as well as transplacental distribution of pindolol are presented.

2. Materials and methods

2.1. Enantioselective analysis of pindolol in amniotic fluid and breast milk

Aliquots of amniotic fluid or maternal milk (500 μL) were added to 50 μL of 1 M NaOH solution and 5.0 mL *tert*-methylbutyl ether. Extraction was performed by shaking for 30 min in a horizontal shaker (220 ± 10 cycles/min) followed by centrifugation at 2000 × *g* for 10 min. Organic phases were evaporated dry, the residues were reconstituted in 100 μL of the mobile phase, and 20 μL samples were submitted to chromatographic analysis.

The HPLC system consisted of a Shimadzu chromatograph (Kyoto, Japan) equipped with a RF-10 AXL fluorescence detector operating at 265 nm (excitation) and 310 nm (emission). The pindolol enantiomers were resolved on a 250 mm × 4.6 mm Chiralpak AD® column (Daicel Chemical Industries, New York, NY, USA) using hexane:ethanol:diethylamine (88:12:0.2, v/v/v) as the mobile phase, at a flow rate of 1 mL/min. The elution order of pindolol enantiomers was obtained by the analysis of individual enantiomers previously separated and collected from the Chiralpak AD® column using hexane:ethanol:diethylamine (88:12:0.2, v/v/v) as the mobile phase, as reported above. The solvent was evaporated dry, and the residues were dissolved in the mobile phase (acetonitrile: 0.3 M aqueous sodium perchlorate 40:60, v/v) and analysed on a Chiralcel OD-R column as described by Zhang et al. [7].

The calibration curves were constructed using drug-free breast milk or amniotic fluid (500 μL) spiked with racemic pindolol to give final concentrations of 1.0–84.0 ng of each pindolol enantiomer/mL breast milk and 5.0–100.0 ng of each pindolol enantiomer/mL amniotic fluid. Recoveries, limit of quantification, linearity, precision, and accuracy were determined as shown in Table 2.

Bench-top stability was investigated using two samples each of amniotic fluid (7.4 and 296.0 ng/mL) and breast milk (7.4 and 56.0 ng/mL) left at room temperature for 6 h. The samples were then processed and analysed. Freeze/thaw stability was evaluated after three cycles of freezing and thawing in aliquots of the same samples used for bench-top stability. Aliquots of the cited samples were also used to evaluate autosampler stability (10 h at 4 °C) and long-term stability (28 days at −20 °C).

2.2. Enantioselective analysis of pindolol in plasma and urine

The enantioselective analysis of pindolol in plasma and urine was conducted as reported previously by Gonçalves et al. [8].

2.3. Method application

2.3.1. Patients and clinical protocol

The study was conducted on six hypertensive pregnant patients aged 25–35 years with a gestational age of 37–40 weeks and with a medical indication for the use of pindolol (Table 1). The patients were selected during the prenatal care period at the University Hospital of the Faculty of Medicine of Ribeirão Preto-USP (HCFMRP-USP). The patients were informed in detail about the research project and gave written informed consent to participate. The research project was approved by the Research Ethics Committee of HCFMRP-USP (Process 6842/2001).

The patients were treated with one 10 mg pindolol tablet every 12 h for at least 3 days. On the day of delivery the patients were admitted to the Obstetric Center of HCFMRP-USP for collection of biological material. Blood samples, approximately 5 mL, were collected through an intravenous catheter at 0, 0.5, 1, 1.5, 2, 4, 6, 8, 10, and 12 h after the administration of the last pindolol dose, and urine was collected at intervals of approximately 3 h. Amniotic fluid was collected at the time of resolution of delivery and maternal milk was collected after delivery (Table 1).

Table 1
Individual characteristics of the patients ($n = 6$) treated with racemic pindolol (10 mg/12 h)

Patient	Age (years)	Fetal gestational age (weeks)	Weight before delivery (kg)	Amniotic fluid[a] (h)	Milk[a] (h)
1	25	37	100.0	9.0	–
2	28	38	98.8	8.0	12
3	33	40	88.0	4.0	14
4	30	39	79.0	2.5	–
5	35	40.2	68.0	1.7	11
6	28	39	–	3.0	–

[a] Time after the last pindolol dose.

Heparin was added to blood as an anticoagulant (Liquemine® 5000 IU, Roche). Blood samples were centrifuged at 2000 × *g* for 15 min, and plasma was stored at −70 °C. Total urine volume was determined at each interval and an aliquot was stored at −70 °C, and amniotic fluid and maternal milk were also stored at −70 °C until the time for chromatographic analysis.

2.3.2. Pharmacokinetic analysis

The pharmacokinetics of pindolol enantiomers was calculated within one dose interval by fitting the data to a one-compartment model. Pharmacokinetic parameters were determined as previously described [8].

2.3.3. Statistical analysis

Data were analysed statistically by the Wilcoxon test for paired nonparametric values, with the level of significance set at $p < 0.05$. The data are expressed as median and range.

3. Results and discussion

The investigation of stereoselectivity of pindolol in maternal/amniotic fluid transfer and its excretion into breast milk requires the availability of sensitive and selective analytical methods.

Recoveries of pindolol enantiomers from amniotic fluid and breast milk were greater than 90% (Table 2) using methyl *tert*-butyl ether as the extracting solvent. After plasma alkalinization (pH 9.0), liquid–liquid extractions have been reported using ethyl acetate [6,17], diethyl ether [12–14], or methyl *tert*-butyl ether [8] with recoveries higher than 75%. Zhang et al. [7] employed diol (2OH) solid-phase extraction cartridges with similar recoveries of 72% for both enantiomers from plasma. Mangani et al. [15] described an on-line sample clean-up performed using a restricted access cartridge and reported satisfactory recoveries from serum (>90%). A precolumn packed

Table 2
Confidence limits for the enantioselective analysis of pindolol in amniotic fluid and breast milk samples

	Amniotic fluid		Breast milk	
	(+)-*R*-Pindolol	(−)-*S*-Pindolol	(+)-*R*-Pindolol	(−)-*S*-Pindolol
Recovery, % ($n = 3$)	96.7 (6.1)	98.7 (8.8)	92.1 (8.6)	91.9 (6.2)
Linearity				
Range (ng/mL)	1.0–1000.0	1.0–1000.0	1.0–84.0	1.0–84.0
Correlation coefficient (r^2)	0.999	0.999	0.995	0.996
Quantitation limit ($n = 5$)				
Concentration (ng/mL)	1.0	2.0	1.05	1.05
Intra-assay precision (CV%)	11.2	13.9	13.8	13.6
Intra-assay accuracy (%)	−3.0	13.7	10.5	8.6
Intra-assay precision, CV% ($n = 10$)				
27.5 ng/mL (amnion) 4.2 ng/mL (milk)	9.9	8.9	14.4	14.9
55 ng/mL (amnion) 26.2 ng/mL (milk)	1.9	4.6	8.6	6.9
110 ng/mL (amnion) 52.4 ng/mL (milk)	4.5	4.1	9.1	9.8
Inter-assay precision, CV% ($n = 5$)				
27.5 ng/mL (amnion) 4.2 ng/mL (milk)	7.5	8.2	7.2	5.9
55 ng/mL (amnion) 26.2 ng/mL (milk)	7.8	7.2	6.8	8.6
110 ng/mL (amnion) 52.4 ng/mL (milk)	7.6	3.9	3.7	8.2
Intra-assay accuracy, R.E.% ($n = 10$)				
27.5 ng/mL (amnion) 4.2 ng/mL (milk)	9.9	9.4	10.0	15.2
55 ng/mL (amnion) 26.2 ng/mL (milk)	14.8	15.0	7.5	14.0
110 ng/mL (amnion) 52.4 ng/mL (milk)	14.5	13.8	14.6	14.7
Inter-assay accuracy, R.E.% ($n = 10$)				
27.5 ng/mL (amnion) 4.2 ng/mL (milk)	0.5	−0.4	5.5	5.2
55 ng/mL (amnion) 26.2 ng/mL (milk)	7.8	10.0	0.1	2.6
110 ng/mL (amnion) 52.4 ng/mL (milk)	7.6	10.3	3.9	2.8

with a silica-based-cation-exchanger was used by Motoyama et al. [16] for on-line serum and urine clean-up with recoveries higher than 94%.

The quantification limits of 1.0 ng/mL for both enantiomers (Table 2) were obtained with the extraction of amniotic fluid and breast milk aliquots of only 500 μL. The quantitation limits reported for serum and urine using a chiral column and HPLC with fluorescence detection were 0.5 and 5.0 ng/mL, respectively [8]. Lower quantitation limits (0.25 ng/mL plasma or urine) have been reported only with the use of LC–MS–MS [16]. The quantitation limits obtained in the present study are fundamental for the determination of the low concentrations found up to 12 h after the administration of racemic pindolol in pregnant patients.

The data presented in Table 2 show that the methods are precise and accurate. The coefficients of variation and the relative errors obtained were lower than 15% for all concentrations tested. The data obtained in the precision and accuracy study also demonstrated that an internal standard is not required in the analysis.

The tests of bench-top (6 h at room temperature), long-term (28 days at −20 °C), freeze/thaw (3 cycles) and autosampler (10 h at 4 °C) stabilities insured the good condition of the samples during freezing, from collection to analysis, with no variation of more than 15% at any of the concentrations tested.

Pindolol enantiomers were detected in all samples of amniotic fluid collected from the six patients investigated and treated with racemic pindolol (10 mg/12 h); Fig. 1. The data showed wide inter-patient variability with concentrations of 1.2–22.6 ng/mL for (+)-*R*-pindolol and 1.9–15.8 ng/mL for (−)-*S*-pindolol, with no stereoselectivity being observed ($p > 0.05$, Wilcoxon test); Table 3. The ratios of amniotic fluid/maternal plasma concentrations ranged from 0.4 to 4.5 for (+)-*R*-pindolol and from 0.5 to 3.5 for (−)-*S*-pindolol in samples collected within an interval of 1.7–9.0 h after the administration of the last dose of pindolol, and therefore still within a steady-state (dose interval of 12 h). These preliminary results suggest that lower protein binding of pindolol enantiomers (40–60%) may be associated with higher amniotic fluid concentrations. It should also be pointed out that the pH of amniotic fluid is reported to be 7.13, i.e., lower than plasma pH and possibly able to concentrate weak bases such as pindolol [18]. Loughhead et al. [9] reported amniotic fluid to maternal serum ratios of 172% for venlafaxine. The authors also reported no significant correlation between the concentrations of antidepressants in maternal serum and amniotic fluid, suggesting the involvement of more complex variables. Amniotic fluid concentrations of labetalol 2–3 h after dosing were reported to be lower than that in plasma [19].

In the present study, milk-to-plasma concentrations for (+)-(*R*)-pindolol and (−)-(*S*)-pindolol ranged from 0.6 to 3.7 and from 1.1 to 2.8, respectively (Table 3). The excretion of pindolol enantiomers into breast milk was not enantioselective (Fig. 1). Shannon et al. [20] reported the excretion of several beta blockers into breast milk. The authors reported that, on an average, milk-

Table 3
Pharmacokinetic parameters of pindolol enantiomers in parturient women ($n = 6$) treated with multiple doses of racemic pindolol (10 mg/12 h)

	(+)-*R*-Pindolol	(−)-*S*-Pindolol
Plasma		
C_{max} (ng/mL)	13.4 (9.6–37.2)	16.6 (11.0–36.9)
t_{max} (h)	1.5 (1.0–2.0)	1.5 (1.0–1.8)
$t_a^{1/2}$ (h)	0.5 (0.3–1.7)	0.7 (0.2–1.4)
K_a (h^{-1})	1.4 (0.4–2.1)	1.1 (0.5–4.1)
$t^{1/2}$ (h)	3.0 (0.7–4.5)	2.8 (0.9–3.1)
K_{el} (h^{-1})	0.23 (0.2–1.0)	0.25 (0.2–0.7)
AUC_{ss}^{0-12} (ng h/mL)	70.1 (52.6–100.5)	76.7 (55.2–103.7)
Cl/f (L/h)	72.0 (49.8–95.1)	65.6 (48.2–90.6)
Vd/f (L)	377.4 (47.8–427.7)	245.7 (73.0–410.6)
Urine		
Cl_R (L/h)	3.7 (2.7–24.9)	4.8 (2.8–28.2)
Fel (%)	6.6 (3.1–26.2)	6.5 (1.2–31.2)
Ae (μg)	332.1 (155.9–1310.5)	474.2 (192.9–1558.6)
Transplacental transfer		
Umbilical cord (ng/mL)	3.1 (2.5–3.2)	3.5 (2.3–9.9)
Maternal plasma (ng/mL)	3.0 (0.6–17.9)	4.3 (0.7–15.4)
Umbilical cord/maternal plasma	0.6 (0.5–1.2)	0.7 (0.6–1.2)
Amniotic fluid		
Amniotic fluid (ng/mL)	10.1 (1.15–22.57)	10.9 (1.9–15.76)
Maternal plasma (ng/mL)	3.0 (0.6–17.9)	4.3 (0.7–15.4)
Amniotic fluid/maternal plasma	1.86 (0.41–4.47)	2.6 (0.51–3.47)
Breast milk		
Breast milk (ng/mL)	1.9 (1.2–4.2)	3.1 (1.5–3.9)
Maternal plasma	1.1 (0.9–3.2)	1.4 (1.4–2.5)
Breast milk/maternal plasma	1.4 (0.6–3.7)	1.2 (1.1–2.8)

Data are reported as median and (range). $^{*}p < 0.05$, Wilcoxon test.

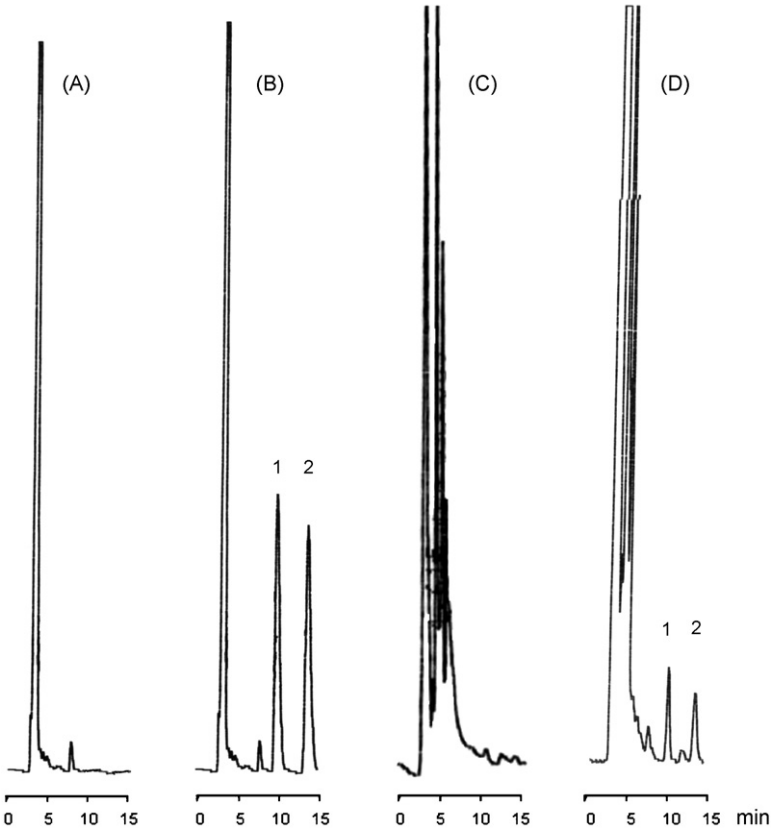

Fig. 1. Chromatograms: (A) blank of aminiotic fluid; (B) amniotic fluid sample obtained 4 h after the last dose of racemic pindolol (10 mg/12 h) ((+)-(*R*): 22.6 ng/mL and (−)-(*S*): 14.6 ng/mL); (C) blank of breast milk; (D) breast milk sample obtained 12 h after the last pindolol dose ((+)-(*R*): 4.2 ng/mL and (−)-(*S*): 3.9 ng/mL). (1) (+)-*R*-Pindolol (11.6 min) and (2) (−)-*S*-pindolol (14.2 min).

to-serum concentrations ranged from 3.0 to 3.5 for metoprolol, 4.6 for sotalol, 2.7 for nadolol, from 2.0 to 11.6 for betaxolol, 2.0 for propranolol, and from 1.1 to 3.1 for atenolol.

The disposition of pindolol in maternal plasma (Fig. 2) and urine was not enantioselective in the six patients investigated (Table 3). Gonçalves et al. [8] reported higher AUC and renal clearance values for (−)-*S*-pindolol in nine patients with pregnancy-induced hypertension, although AUC *S*/*R* ratio (1.1) and renal clearance *S*/*R* ratio (1.2) were close to unit. These findings are similar to those reported by Hsyu and Giacomini [5] who found AUC *S*/*R* ratio in healthy volunteers also close to unit (1.1). With regard to the renal clearance of pindolol enantiomers, *S*/*R* ratios of 1.2 and 1.3, respectively, were reported by Hsyu and Giacomini [5] and Somogyi et al. [13] in the investigation of healthy volunteers. In the present study, we found renal clearance *S*/*R* ratio of 1.3, although with no difference between the enantiomers ($p > 0.05$) probably due to the high data variability (Table 3) and the small number ($n = 6$) of investigated patients. Transplacental transfer of pindolol enantiomers at delivery was also not enantioselective. Plasma cord concentrations of pindolol enantiomers were close to maternal plasma concentrations (umbilical cord/maternal plasma ratios ∼0.7) (Table 3).

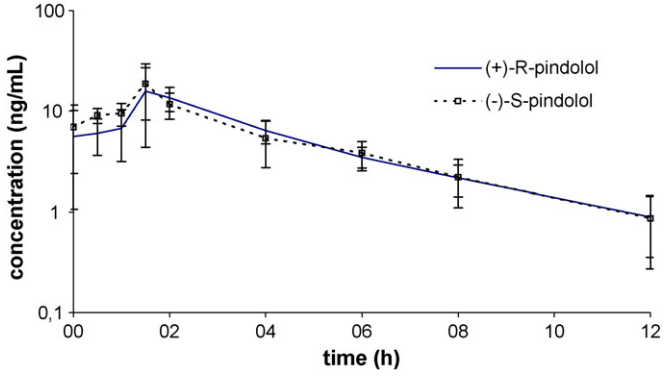

Fig. 2. Plasma concentrations vs. time curves for (+)-*R*-pindolol and (−)-*S*-pindolol after the last dose of racemic pindolol (10 mg/12 h). Data are shown as mean ± S.D.

4. Conclusions

The methods developed and validated for the analysis of pindolol enantiomers in amniotic fluid and maternal milk are sensitive, precise, and accurate and can be applied to clinical pharmacokinetic studies. The investigation of six pregnant and lactating women treated with racemic pindolol (10 mg/12 h) suggested accumulation of both pindolol enantiomers in amniotic fluid (amniotic fluid/maternal plasma ∼2.0) and in maternal milk (milk/maternal plasma ∼1.3).

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