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Resolution of indobufen enantiomers by capillary zone electrophoresis Pharmacokinetic studies of human serum

Franciszek K. Główka*, Marta Karaźniewicz

Department of Physical Pharmacy and Pharmacokinetics, University of Medical Sciences, 6 Święcickiego Street, 60-781 Poznań, Poland

Abstract

A direct and stereospecific method was worked out to quantify indobufen enantiomers in human serum using capillary zone electrophoresis (CZE). The indobufen enantiomers and (+)-*S*-ketoprofen (internal standard, IS) were separated in a fused silica capillary, filled with heptakis 2,3,6-tri-*O*-methyl-β-cyclodextrin as a chiral selector in a buffer of pH 5.0. Indobufen enantiomers and other non-steroidal anti-inflammatory drugs: flurbiprofen, ketoprofen and (+)-*S*-naproxen were also separated during one analytical run. UV absorbances of indobufen enantiomers were measured at 282 nm. Influence of temperature on resolution of the enantiomers, and the electrophoretic parameters: electrophoretic (μ_{ep}) and electroosmotic (μ_{EOF}) mobilities were also determined. Validation of the method was carried out. Calibration curves of indobufen enantiomers were linear in the range of 0.2–20.0 μg/ml. Percent recovery of both enantiomers from acidified serum was calculated after extraction with methylene chloride. Intra- and inter-day measurement precision and accuracy were below 15.0%. Limits of quantitation and detection were also estimated. The elaborated method was tested in vivo after administration of a single dose of 200 mg *rac*-indobufen tablets to healthy volunteers. Calculated parameters confirmed usefulness of the method in human pharmacokinetic studies on indobufen enantiomers. The direct CZE method can provide an alternative to HPLC, where enantiomers used to be derivatised before determination.

Keywords: Enantiomer separation; Non-steroidal; Anti-inflammatory drugs; Indobufen; Ketoprofen

1. Introduction

Indobufen (INDB) (2-[*p*-(1-oxo-2-isoindolinyl)-phenyl]-butyric acid) belongs to the group of non-steroidal anti-inflammatory drugs (NSAIDs). It is able to inhibit tromboxane production and cyclooxygenase (COX)-dependent platelet aggregation, blocking the arachidonic acid metabolism at the level of COX. The effect of INDB on platelet function is transient and reversible, compared with the irreversible inhibition caused by aspirin. INDB possesses an asymmetric carbon (Fig. 1) and exists in two enantiomeric forms as (−)-*R* and (+)-*S*. Its structure is similar to that of derivatives of 2-arylpropionic acid but profens α-methyl group is replaced by an α-ethyl group. Up to now, INDB has been used in the medical treatment as a racemate, though its anti-platelet and anti-inflammatory activity resides mainly in (+)-*S*-enantiomer (eutomer) [1–4]. After administration of *rac*-INDB to healthy volunteers [5,6] or patients with obliterative atherosclerosis [7], serum levels of the (+)-*S* enantiomer were significantly lower than those of the (−)-*R* antipode. It resulted from weaker association of (+)-*S*-enantiomer with serum proteins and, therefore, its faster elimination [8]. Moreover, INDB is completely absorbed from GI tract [9] and its enantiomers do not undergo metabolic chiral inversion [6]. Up to now, reversed phase HPLC has been mainly used for determination of INDB enantiomers as L-leucinamide diastereoisomers after prior diethyl ether extraction [6] or solid-phase extraction (SPE) procedure in columns with C_{18} stationary phase [10]. The direct techniques, including separation in chiral columns, have also been used [11]. The aim of the publication was elaboration and validation of capillary zone electrophoresis (CZE) method for determination of INDB enantiomers in human serum, as an alternative to HPLC, designed for pharmacokinetic and bioavailability studies.

* Corresponding author. Tel.: +48-61-869-91-81x535; fax: +48-61-865-70-05.
E-mail address: glowka@amp.edu.pl (F.K. Główka).

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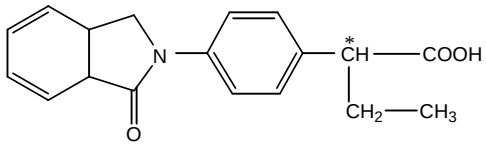

Fig. 1. Chemical structure of indobufen ((∗) chiral center).

2. Experimental

2.1. Materials

rac-INDB (mp 183 °C) and (+)-*S*-INDB (mp 198 °C, optical purity (o.p.) 99.6%) were obtained from Pharmacia & Upjohn (Milan, Italy). *rac*-Ketoprofen (*rac*-KTP) (mp 94–97 °C), (+)-*S*-KTP (mp 75–78 °C, o.p. 99.0%), (+)-*S*-naproxen [(+)-*S*-NPX, mp 154–155 °C, o.p. 98.0%], *rac*-flurbiprofen (*rac*-FBP) (mp 110–112 °C), (+)-*S*-FBP (mp 109–110 °C, o.p. 98.0%) and heptakis 2,3,6-tri-*O*-methyl-β-cyclodextrin (TMβCD) were purchased from Sigma (St. Louis, MO, USA). Eighty-five percent orthophosphoric acid (P.O.Ch., Gliwice, Poland) and triethanolamine (Applied Science Laboratories, State College, PA, USA), 1.0 M and 0.1 M NaOH (Agilent Technologies, Waldbronn, Germany) were used. Methanol (Merck, Darmstadt, Germany) was of HPLC grade. Demineralised water (0.1 μS/cm) was always used (Seradest USF 1900, USF Seral, Germany).

2.2. Apparatus and CE conditions

Separation of INDB enantiomers was performed in an Agilent model ^{3D}CE apparatus (Agilent Technologies) with UV detector set at $\lambda_{max} = 282$ nm. Serum samples, after extraction and dissolution in a mixture of methanol-water (1:3), were hydrodynamically injected at the anode. An Agilent fused silica capillary of 50 μm i.d. with a lightpath three times extended and of a 70 cm total length (effective length of 61.5 cm) was used in the experiment. Temperature of the capillary was maintained at 25 °C by a thermostatic system. Separations were performed at 20 kV voltage and 50 × 5 mbar s injection (5.5 nl injected volume). The apparatus was equipped with ChemStation used for an instrument control, data acquisition and data analysis. Windows NT software was used to drive the system.

A new capillary was flushed with 1.0 M NaOH, 0.1 M NaOH, water and background electrolyte (BGE) for 10, 10, 5 and 8 min, respectively. Prior to each run the capillary was rinsed out with 0.1 M NaOH, demineralised water and BGE with chiral selector for 10, 6 and 12 min, respectively.

The INDB enantiomers were determined in a 0.02 M triethanolamine-phosphate buffer (BGE) of pH 5, with TMβCD. To receive the buffer, stock solutions of triethanolamine and ortophosphoric acid, with 0.2 M concentrations each, were prepared. The volume of 1.4 ml triethanolamine was diluted in demineralised water, bringing it up to 50 ml (I) and 1.35 ml of 14.8 M orthophosphoric acid was also diluted with demineralised water bringing the volume up to 100 ml (II). The buffer was prepared by mixing 1 ml volume of I with 1 ml of II solution and by adding demineralised water up to 10 ml. Final pH of the buffer was approximately 5.0. The appropriate amount of 71.45 mg of a chiral selector TMβCD was dissolved in 1 or 2 ml volume of the electrolyte to receive 0.025 M or 0.05 M solutions, respectively. The solution was passed through a filter of 0.45 μm pore size and degassed by ultrasounds before injection into the capillary.

2.3. Influence of temperature on enantiomer separation

INDB enantiomer separation was investigated at the temperatures of 10, 15, 20 and 25 °C, maintained in the capillary by a thermostatic system to receive optimal resolution of analytes. However, in previous studies enantiomer separation was carried out at the temperature of 35 °C [12].

2.4. CE calculations

Electroosmotic flow (μ_{EOF}) was calculated from an equation:

$$\mu_{EOF} = \frac{lL}{t_{eo}U}$$

where l denotes capillary length between the injection end and the detector (cm), L is the capillary overall length (cm), t_{eo} the migration time for a peak of electroosmotic flow (s), and U is the applied voltage.

The apparent electrophoretic mobilities (μ_{ap}) of the enantiomers were also estimated from the above equation, using the migration time for each analyte instead of t_{eo}. The effective electrophoretic mobilities (μ_{ep}) were calculated from the following equation of apparent electrophoretic mobility for the ion:

$$\mu_{ap} = \mu_{ep} + \mu_{EOF}$$

The resolution (R_s) was calculated from:

$$R_s = \frac{2(t_{migrR} - t_{migrS})}{w_1 + w_2}$$

where $t_{migrR,S}$ are migration times of (+)-*S*- and (−)-*R*-INDB, $w_{1,2}$ are widths at the peak base.

Relative migration time:

$$t_{S,R} = \frac{t_{migrR,S}}{t_{IS}}$$

where t_{IS} is the migration time of the internal standard (IS).

Chiral selectivity:

$$\alpha = \frac{t_{migrR} - t_{eo}}{t_{migrS} - t_{eo}}$$

2.5. Calibration curve of INDB enantiomers in human serum

INDB and IS stock solutions of 1 mg/ml each were prepared by dissolving an appropriate amount of *rac*-INDB and (+)-*S*-KTP in methanol. Then, standard solutions of 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, 100.0 and 200.0 μg/ml *rac*-INDB and 100.0 μg/ml (+)-*S*-KTP were prepared also in methanol. The volume of 100 μl of the sample was transferred to a 4 ml screwed cup glass vial containing 0.5 ml blank serum. The resulting serum contained, respectively, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 μg/ml of each INDB enantiomer and 20.0 μg/ml (+)-*S*-KTP. The samples were processed according to the procedure specified below.

2.6. Procedure of preparation of serum samples

Serum samples with INDB enantiomers and IS were acidified with 0.2 ml 1 M orthophosphoric acid and 2 ml of methylene chloride was added. The mixture was shaken for 10 min and, then, cooled at 4 °C. It was centrifuged for 5 min and the lower layer was transferred to a clean glass tube and evaporated to dryness at 40 °C under a gentle flow of nitrogen. The residue was reconstituted in 50 μl of methanol and 150 μl of water and the volume of 5.5 nl was injected into the capillary. Samples of serum from volunteers, who received a 200 mg *rac*-INDB tablet, were processed in the same manner, except that each 0.5 ml serum was spiked with 100 μl IS.

2.7. Validation parameters

2.7.1. Linearity

Linearity of the calibration curve was estimated for the peak area of INDB enantiomer to IS ratio as a function of the analyte concentration in serum samples, covering the range of 0.1–20.0 μg/ml. The equations of calibration curves were used to calculate unknown INDB enantiomer concentrations in human serum. A correlation coefficient was calculated to confirm linearity of the calibration curve.

2.7.2. Limit of detection (LOD) and limit of quantitation (LOQ)

LOD was determined as a signal to noise baseline ratio (S/N) of 4:1. LOQ is defined as the lowest concentration of INDB enantiomer of the calibration curve within the R.S.D. ≤ 15.0% of its nominal value.

2.7.3. Precision and accuracy

Intra-day precision of the elaborated method was calculated for 1.0 and 10.0 μg/ml INDB enantiomer concentrations in human serum, for five samples of each concentration. Inter-day precision was estimated for all concentrations within the calibration curve range. The precision was expressed as R.S.D. [=(S.D./mean concentration) × 100]. Accuracy was estimated for the same range of enantiomer concentrations as for evaluation of precision of the method. It was expressed by the percent difference between the mean concentration determined and the nominal concentration: error (%) = [(mean concentration − nominal concentration)/nominal concentration] × 100.

2.7.4. Recovery

The recovery of 1.0 and 10.0 μg/ml INDB enantiomer concentrations were calculated. First series consisted of five 0.5 ml blank serum samples spiked with 100 μl of 10.0 or 100.0 μg/ml *rac*-INDB and 100 μl of 100 μg/ml IS each. The samples were extracted according to the above procedure. Then, five blank serum samples of second series were supplemented with IS only. INDB enantiomers were added to a dry residue after extraction. The recoveries were calculated as the ratio of peak area for each extracted or non-extracted INDB enantiomer (1.0 and 10 μg/ml) to peak area of IS.

2.8. In vivo tests—pharmacokinetic analysis

Usefulness of the elaborated method of INDB enantiomer determination in pharmacokinetic and bioavailability studies on INDB enantiomers was tested in vivo. The studies were approved by the Human Investigation Ethical Committee at the University of Medical Sciences in Poznań. A single dose of 200 mg *rac*-INDB was administered to healthy volunteers. Blood samples (4–5 ml) were obtained (in serum gel tubes S/4.7 ml, Sarstedt, Monovette, Germany) at the following times: immediately before administration and at 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 9.0 and 12.0 h after administration. Within 30 min following blood withdrawal, the samples were centrifuged and kept frozen in plastic vials at −27 °C until analyzed. The serum INDB enantiomer concentrations were used to calculate pharmacokinetic parameters. The Topfit 2.0 software package was used for the calculation. Area under the curve ($AUC_{0\rightarrow\infty}$) was estimated by trapezoidal rule with extrapolation to infinity using the ratio C_n/k_{el} where C_n was the last measurable concentration. The elimination rate constant (k_{el}) was estimated from the terminal linear segment of the log serum concentration/time data. The elimination half life ($t_{0.5}$) was calculated from $\ln 2/k_{el}$; t_{max} was estimated from an enantiomer concentration/time curve and C_{max} was read at t_{max}. Clearance of the drug from serum (Cl) was calculated dividing the dose (D) of each enantiomer by $AUC_{0\rightarrow\infty}$ (assuming complete bioavailability). Volume of distribution (V_d) was estimated from $D/AUC_{0\rightarrow\infty}$ and mean residence time (MRT)—from AUMC/AUC (AUMC—area under the first moment curve).

3. Results and discussion

3.1. CE conditions of INDB enantiomer resolution

In earlier studies on separation of enantiomers of derivatives of 2-arylpropionic acid, using CZE technique no

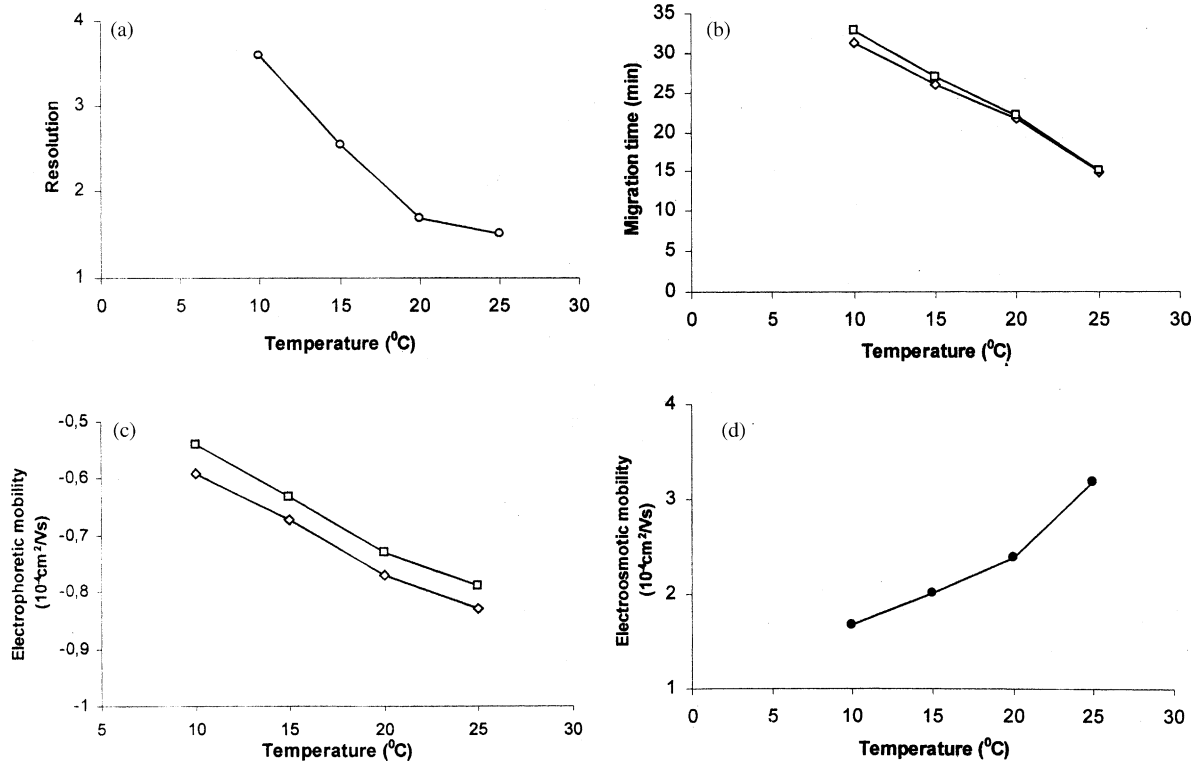

Fig. 2. Effect of temperature on (a) resolution, (b) migration time, (c) electrophoretic mobility of INDB enantiomers and (d) electroosmotic mobility. BGE: 20 mM triethanolamine phosphate buffer, pH 5.0, 25 mM TMβCD (chiral selector), 70 cm total length of the capillary, voltage 20 kV, current 5.4–7.4 μA. (□) (+)-*S*-INDB; (◇) (−)-*R*-INDB.

separation of INDB enantiomers could be obtained [12], even if TMβCD, the best chiral selector for separation 2-APA [13,14] was used. Therefore, in order to define optimum conditions for INDB enantiomer separation, effects of temperature on individual CE parameters were examined. Temperature in the capillary affects viscosity of BGE, its pH and affinity of the compound to cyclodextrins: the constant of complex stability shows values decreasing with an increase in temperature [15,16]. The obtained results showed that an increase in the temperature was accompanied by decreasing values of the resolution, R_S, from 3.59 at 10 °C to 1.5 at 25 °C (Fig. 2a). This probably reflected decreasing difference in stability of (−)-*R*- and (+)-*S*-INDB complexes with CDs [13]. Migration times of INDB enantiomers were shortened from around 30 min at 10 °C to around 15 min at 25 °C (Fig. 2b). As evident in Fig. 2c, electrophoretic flow decreased with increasing temperature. It might be the result of a decreased BGE viscosity, though the augmented values of electroosmotic flow were observed (Fig. 2d). The temperature of 25 °C was accepted as the optimum since complete separation of enantiomers could be obtained at the temperature in the relatively short period of time (15 min) (Fig. 3). On the other hand, at the temperature of 35 °C, at which enantiomers of 2-APA derivatives were separated [12], no INDB enantiomer resolution could be obtained. This might reflect extensive diffusion of the analyte in the capillary [13]. Effects of TMβCD concentration were also analyzed on resolution of INDB enantiomers and derivatives of 2-APA in mixture. Using 80 cm fused capillary (longer than for analysis of INDB) filled with 20 mM triethanolamine-phosphate buffer of pH 5, containing 50 mM TMβCD, enantiomer derivatives of 2-APA (NPX, FBP, KTP) and INDB were separated. The time required to separate seven enantiomers peaks exceeded 40 min (Fig. 4). On the other hand, the concentration of 25 mM TMβCD was sufficient for a complete separation of INDB enantiomers following extraction from human serum and the separation required less time (Fig. 3). Peaks of 2-APA derivatives did not separate from each other using 25 mM concentration of TMβCD at the temperature of 35 °C. Identity of enantiomer peaks of analyzed compounds appearing in the electropherogram was checked injecting sequentially the enantiomer standards. The sequence of demonstrated INDB peaks was reverse to that for 2-APA derivatives. In the electropherogram (+)-*S* peak appeared first, followed by (−)-*R*-INDB peak (Figs. 3 and 4). The complete separation of INDB enantiomers obtained at 25 °C using 25 mM TMβCD and the absence of peaks originating from endogenous compounds in the electropherogram of a serum extract pointed to stereospecificity of the designed technique. Taking under consideration the mentioned above different properties of INDB or 2-APA enantiomers and in consequence different CZE separation conditions it does not seem to be possible to significantly lower migration times of the mentioned chiral compounds without affecting

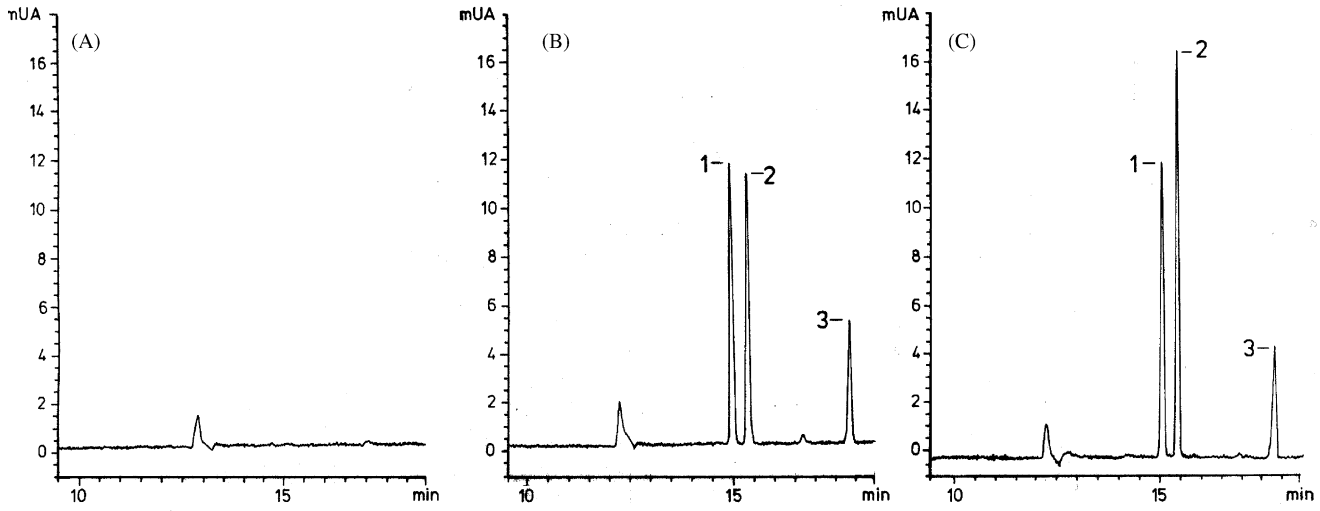

Fig. 3. Electropherograms obtained after extraction of human serum samples: (A) blank serum; (B) serum spiked with the concentration of 10 μg/ml of (+)-*S*- and (−)-*R*-INDB of each enantiomer and 20 μg/ml of (+)-*S*-KTP (internal standard), (C) sample of a healthy volunteer serum at 3.0 h elapsed from the administration of a single dose 200 mg *rac*-INDB tablet. Enantiomer concentrations were 10.4 μg/ml of (+)-*S*-INDB and 14.8 μg/ml of (−)-*R*-INDB. Peaks 1, 2, 3 denote (+)-*S*-INDB, (−)-*R*-INDB and (+)-*S*-KTP, respectively.

the earlier obtained selective separation, presented in Fig. 4.

3.2. Validation parameters

3.2.1. Linearity, LOD and LOQ

Standard curves for INDB enantiomers proved to be linear in the concentration range of 0.2–20.0 μg/ml. Equations of the standard curves are presented in Table 1. The established equations of INDB enantiomer standard curves were used to calculate serum concentrations in volunteers following administration of 200 mg *rac*-INDB tablet. For the worked out technique, LOD (S/N = 4 : 1) was 0.1 μg/ml. On the other hand, LOQ, defined as the lowest concentration in the standard curve at which R.S.D.-defined precision ≤15%, amounted to 0.2 μg/ml.

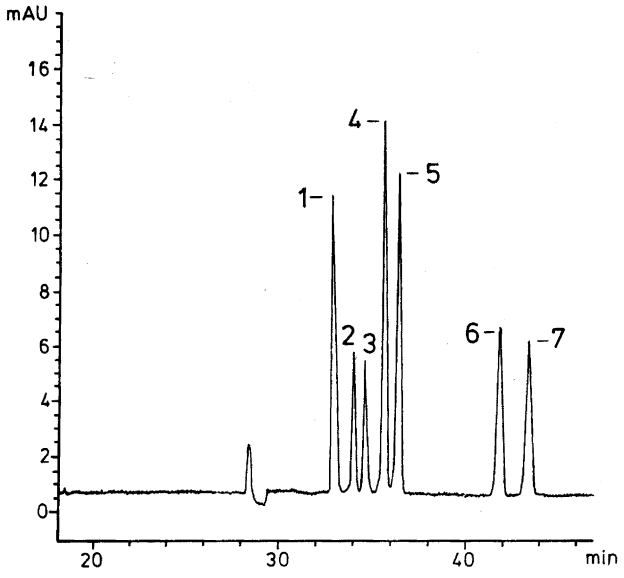

Fig. 4. Electropherogram of the enantiomeric separation of INDB and 2-APA derivatives spiked into a capillary as a mixture in methanol-water (50:150, v/v); (+)-*S*-NPX (1); (−)-*R*-FBP (2); (+)-*S*-FBP (3); (+)-*S*-INDB (4); (−)-*R*-INDB (5); (−)-*R*-KTP (6); (+)-*S*-KTP (7). Conditions of separation: 80.5 cm total length of the capillary, filled with 20 mM triethanolamine–phosphate buffer of pH 5 (BGE), containing 50 mM TMβCD; temperature of determination 20 °C.

3.2.2. Precision, accuracy and recovery

The designed technique was characterized by high precision of estimation of INDB enantiomer concentrations both within a day and between days, as proved by low values of the R.S.D. (<15%). Also the accuracy of estimations fitted the range accepted for techniques of drug testing in body fluids and amounted to ≤15% (Table 1). Recovery of serum INDB enantiomers following extraction with dichloromethane was relatively high, ranging between 86 and 94% and was comparable to that in SPE extraction (above 90%) [10].

3.3. Pharmacokinetic studies

Administration of a single tablet of 200 mg *rac*-INDB was followed always by lower (+)-*S*-INDB enantiomer serum concentrations than those of (−)-*R* enantiomer. INDB enantiomers were rapidly adsorbed from tablets in GI tract and reached maximum serum levels, $C_{\max\text{-}S} = 15.1 \pm 2.2$ μg/ml and $C_{\max(-)\text{-}R} = 18.9 \pm 2.2$ μg/ml after the time of $t_{\max} = 2.5 \pm 1.5$ h. The relatively low distribution volume for the two enantiomers (around 10 l) resulted probably from very strong binding of the drug by blood proteins (>99%). Also pharmacokinetic parameters: AUC and MRT calculated for each of the enantiomers reached

Table 1
Validation parameters of standard curves for analysis of INDB enantiomers in human serum

Nominal concentration of enantiomer (μg/ml)	(+)-*S*-INDB			(−)-*R*-INDB		
	Mean assayed value (μg/ml)	Accuracy (error, %)	Precision (R.S.D., %)	Mean assayed value (μg/ml)	Accuracy (error, %)	Precision (R.S.D., %)
Intra-day repeatability ($n = 5$)						
1.00	1.14	14.2	11.4	1.13	13.0	10.4
10.00	10.74	7.4	3.8	10.75	7.5	4.8
Inter-day reproducibility ($n = 5$)[a]						
0.20	0.23	15.0	7.7	0.23	15.0	9.7
0.50	0.53	6.0	4.8	0.51	2.8	4.5
1.00	1.10	10.4	5.9	1.11	10.8	4.1
2.00	2.24	12.0	9.2	2.22	11.2	8.5
5.00	5.11	2.1	4.0	5.12	2.5	3.5
10.00	10.64	6.4	2.6	10.64	6.4	2.0
20.00	19.62	1.9	0.9	19.60	2.0	0.6

Linear equations: $y = 0.178x \pm 0.039$, r = 0.9993; $y = 0.177x \pm 0.038$, $r = 0.9992$.
[a] Consecutive calibration curves were prepared in a period of 3 weeks.

Table 2
Pharmacokinetic parameters of INDB enantiomers in two healthy volunteers after administration of 200 mg *rac*-INDB tablet

Volunteer number	Enantiomer	C_{max} (μg/ml)	t_{max} (h)	$AUC_{0\rightarrow\infty}$ (μg h/ml)	MRT (h)	Cl/F (ml/min)	V_d (l)	$t_{0.5}$ (h)
1	(+)-*S*	12.9	4.0	84.7	6.0	39.4	10.5	3.1
	(−)-*R*	17.0	4.0	132.2	7.5	25.2	8.9	4.1
2	(+)-*S*	17.2	1.0	98.2	5.8	34.0	10.9	3.7
	(−)-*R*	20.9	1.0	147.4	7.2	22.6	9.1	4.6
Mean ± S.E.M.	(+)-*S*	15.1 ± 2.2	2.5 ± 1.5	91.4 ± 6.8	5.9 ± 0.1	36.7 ± 2.7	10.7 ± 0.2	3.4 ± 0.3
	(−)-*R*	18.9 ± 2.0	2.5 ± 1.5	139.8 ± 7.6	7.4 ± 0.1	23.9 ± 1.3	9.0 ± 0.1	4.3 ± 0.3

Differences between enantiomers of INDB. NS: nonsignificant, S: significant ($0.01 \leq P < 0.05$), HS: highly significant ($0.001 \leq P < 0.01$).

lower values for (+)-*S*-INDB (Table 2). As mentioned earlier, this resulted from the more rapid elimination of (+)-*S* enantiomer (Cl = 36.7 ± 2.7 ml/min; $t_{0.5} = 3.4 \pm 0.3$ h) as compared to (−)-*R* enantiomer (Cl = 23.9 ± 1.3 ml/min; $t_{0.5} = 4.3 \pm 0.3$ h). The more rapid elimination of (+)-*S* enantiomer might be associated with its weaker binding to serum proteins and to its displacing from complexes with proteins by (−)-*R* enantiomer [8]. Moreover, INDB enantiomers do not undergo chiral inversion [6], often observed for some 2-arylpropionic acid derivatives, mainly for ibuprofen enantiomers or limited inversion for ketoprofen [17].

4. Conclusion

The elaborated CZE method is stereospecific, adequately accurate and precise. It can be an alternative method to HPLC in pharmacokinetic and bioavailability studies on INDB enantiomers.

The conclusion is supported by the fact that the elaborated CZE method requires no pre-capillary derivatization. However, in HPLC method mostly used in pharmacokinetic studies, INDB enantiomers have been determined only as L-leucinamide diastereoisomers in a column with reversed-phase C_{18} [6,10]. The derivatization procedure of INDB has considerably extended serum sample analysis time. Moreover, in HPLC with normal phases, a very expensive chiral stationary phase has been used for determination of INDB enantiomers in pharmaceutical formulations [11]. Injection volume of 5 nl, used in the elaborated CZE method, is considerably smaller as compared to that used in HPLC, where usually 10 μl volume is injected. Small volume allows for a repetitive analysis of prepared serum samples. Nevertheless, despite the differences in an injection volume, the limit of quantitation for both validated HPLC (0.25 μg/ml) [10] and CZE (0.2 μg/ml) methods have been nearly identical. The HPLC method provides the possibility to receive more reproducible retention time than migration times in CZE, but this does not significantly influence results of enantiomer determination. Buffer volume for one analytical run in CZE is smaller than 1 ml whereas HPLC consumes about 20 ml organic/water mobile phase [6,10]. Therefore, the former method is more friendly for environment.

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