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LC procedure with SPE for quantification of indobufen enantiomers: pharmacokinetic studies

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Abstract

A rapid and selective liquid chromatography (LC) with solid phase extraction (SPE) to quantify indobufen (INDB) enantiomers is described. The INDB enantiomers and internal standard (racemic flurbiprofen) are extracted from a small volume of acidified serum (0.2 ml) by means of SPE using cartridges with octadecyl chemically bound phase and analysed on reversed phase (RP), C_{18} column with ultraviolet detection at 275 nm. Recovery of both INDB enantiomers was in the range 92.1–94.3%. The mobile phase is composed of acetonitrile-potassium dihydrogen phosphate (pH 4.75;10 mM) (38:62, v/v). The linear range of the standards curves was from 0.25 to 25.00 μg ml^{-1} in serum for both enantiomers. The limit of quantification and detection for both INDB enantiomers in serum were 0.25 μg ml^{-1} (CV $\leq$ 10%), and 0.1 μg ml^{-1}, respectively. Both intra- and inter-day accuracy and precision of the calibration curves were determined and their CV values did not exceed 10%. The validated method has been successfully applied for chiral pharmacokinetics studies of INDB from tablets and intramuscular injections in man.

Keywords: RP-LC; Indobufen; Enantiomers; Racemate; SPE; Precolumn derivatization; Man serum concentration

1. Introduction

Indobufen (INDB), 2-[4-(-1-oxo-isoindolin-2-yl)-phenyl] butyric acid (Fig. 1), is an inhibitor of platelet aggregation acting through cyclooxygenase inhibition and in consequence synthesis of thromboxane. The inhibition is transient and reversible compared to an irreversible one caused by acetylsalicylic acid [1–3]. The INDB butyric acid side chain possesses an asymetric α-carbon and therefore occurs as the (+)*S*- and (−)*R*-enantiomer. Though the above pharmacological activity resides principally in the (+)*S*-enantiomer, INDB racemate is up to now used in medical treatment. After administration of racemic INDB (tablets, intramuscular injections or suspensions), the plasma or serum levels of (+)*S*-enantiomer were markedly lower than those of the (−)*R*-enantiomer [4,5]. After administration of the *S*-enantiomer no detectable levels of the

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R-enantiomer were found either in plasma or urine, showing that no chiral inversion of *S*-INDB to its *R*-antipode occurs in man [4]. The literature of the subject contains few techniques of estimation of INDB enantiomers. The direct HPLC technique for determination of optical purity of (+)*S*-INDB in dosage forms employed resolution on Chiracel columns [6]. Only one technique has been described up to now for determination of the enantiomers in body fluids: in plasma and urine [4]. In the above technique INDB enantiomers were estimated indirectly, in which derivatisation with L-leucinamide, as well as the diethyl ether extraction were employed while S-indoprofen served as an internal standard. The reversed phase HPLC method has been proposed in this paper for determination of INDB enantiomers to be used in pharmacokinetic studies. The solid phase extraction procedure (SPE) on colmns with C18 phase has been successfully employed for isolation of enantiomers from human serum to replace the classical diethyl ether extraction. Moreover, flurbiprofen (FBP), a new internal standard and a lower serum volume (0.2 ml) have been proposed for the determination. The suggested method has been successfully used in studies on pharmacokinetics of INDB enantiomers in healthy subjects and patients.

2. Experimental

2.1. Materials

Racemic INDB and its sodium salt were purchased form Jelfa S.A. (Jelenia Góra, Poland) and Pharmacia (Milan, Italy) where (+)*S*-INDB enantiomer was obtained, too. The optical purity of (+)*S*-enantiomer was 98.5%. Indobufen-Jelfa

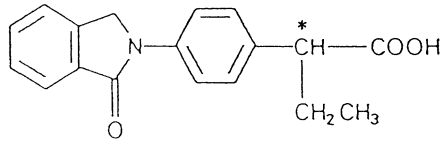

Fig. 1. Chemical formula of indobufen (INDB). *-chiral centre.

intramuscular injections lot number 10395p were prepared in Pharmaceutical Works Jelfa S.A., Ibustrin tablets, lot number A 1061 were commercially obtained (Pharmacia, Italy). Composition of the tablet: indobufen 200 mg, lactose 200 mg, primojel-starch sodium glycolate 50 mg, microcrystalline cellulose 63 mg, magnesium laurylsulfate 2 mg, magnesium stearate 5 mg. Racemic-flurbiprofen (rac-FBP, internal standard, I.S.), m.p. 110–113, ([7]: ≈110°C) was prepared from commercial suppositories (Boots, England) and purified by recrystalization from petroleum ether (P.O.CH, Gliwice, Poland). Acetonitrile and methanol were HPLC grade (Merck, Darmstad, Germany), triethylamine, L-leucinamide, ethyl chloroformate were purchased from Sigma (St Louis, MO), Aldrich (Milwaukee, WI), Merck-Schuchardt, München, Germany), respectively. Potassium dihydrogen phosphate (Xenon, Łódź, Poland) was of reagent grade. House triple distilled water from silica glass equipment was always used.

2.2. Equipment and chromatographic conditions

HPLC determinations of INDB enantiomers in human serum were performed in a Hewlett Packard chromatograph model HP 1100. The mobile phase consisted of acetonitrile–potassium dihydrogen phosphate (pH 4.75; 10 mM) (38:62 v/v) and was filtered through a 0.45 μm silicon membrane filter (Schleicher Schuel, Dassel, Germany), deaireated by degasser model G1322A and pumped by quaternary pump model G1311A at flow rate 1 ml min^{-1}. The detection was measured at $\lambda_{max} = 275$ nm using model G1314A detector with 14 μl high pressure flow cell. Samples (20 μl) were automatically injected using autosampler model G1313A. The chromatographic separation of INBD enantiomers, previously converted to their L-leucinamide diastereoisomers was provided using Purospher 18e (5μm; 125 × 3 mm) column which was protected by Purospher RP 18 (5μm) guard column (both from Merck). HP 1100 machine was equipped with ChemStation used for instrument control, data acquisition and data analysis. The system was controlled by Windows Software.

2.3. Calibration curve

Stock solutions INDB and FBP were prepared with 10 mg ml^{-1} of each in methanol. Then, standard solutions: 1.0, 2.0, 4.0, 10.0, 20.0, 40.0, 80.0 and 100.0 μg ml^{-1} of each INDB enantiomers and 100 μg ml^{-1} of racemic FBP were prepared in methanol. The standard solutions were stable for one month when refrigerated. Then 50 μl each of INDB and I.S. were transferred by automatic pipette to a conical flask (25 ml) containing 0.2 ml blank human serum. The resulting serum containing 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 20.0, 25.0 μg ml^{-1} of each INDB enantiomers and 25.0 μg ml^{-1} racemic I.S. were processed according to the procedure SPE specified below. Limit of detection was determined at a signal to noise of base line in ratio 4:1. The limit of quantification (LoQ) in serum was defined as the lowest concentration on the calibration curve for which assay precision (coefficient of variation, CV) is lower than 10%.

2.4. Solid phase extraction procedure

Cartridges 1 ml capacity with liquid phase C_{18} chemically bound to silica gel (100 mg) were applied for isolation of INDB enantiomers from human serum. The columns were conditioned before use by means of 2×1 ml methanol and 2×1ml triple distilled water. The cartridges were reused several times. Human serum 0.2 ml with INDB enantiomers and I.S. were acidified by 0.75 ml, 0.1 M l^{-1} ortophosphoric acid, shacked and transferred into a SPE column cartridge. Columns with absorbed INDB were purificated by 2×0.5 ml distilled water. The compounds were washed by methanol 300 μl under low vacuum 2–5 mm Hg (flow rate: 30 drops min^{-1}) by using a water pump. Methanol was evaporated to dryness at 70°C at a gentle nitrogen stream.

2.5. Derivatisation procedure

To the evaporated residue 100 μl of 50 mM l^{-1} triethylamine in acetonitryle, followed by, at 30 s intervals, 50 μl of 60 mM l^{-1} ethylchloroformate in acetonitrile, then 50 μl of 1 M l^{-1} L-leucinamide were added. 50 μl of HPLC-grade H_2O was added after 2 min to complete derivatisation [8,9]. A 20 μl aliquot of the solution was used for analysis. Peaks area ratio (−)*R*- or (+)*S*-INDB to I.S. versus their concentrations and the resulting calibration curves were used to calculate unknown (−)*R*- or (+)*S*-INDB concentrations.

2.6. Recovery

INDB enantiomers recovery, for 1 and 10 μg ml^{-1} INDB- enantiomers concentrations were calculated. First series was consisted of five 0.2 ml blank serum spiked with 50 μl of 4 μg ml^{-1} INDB enantiomers standard solution and 50 μl of 100 μg ml^{-1} racemic I. S. solution resulting in 1 μg ml^{-1} of each INDB enantiomers and 25 μg ml^{-1} of the racemic I.S. The samples were processed according to the above SPE. Then to five series serum samples with I.S only (II series) INDB enantiomers were added after the above SPE procedures. Recoveries were calculated as the area ratio of either (−)*R*- or (+)*S*-INDB to I.S. from the spiked samples and unextracted standard solution enantiomers. The recovery for 10 μg ml^{-1} of INDB enantiomers was calculated in the same manner.

2.7. Application of the method in the in vivo conditions–pharmacokinetic analysis

The utility of the worked out method was demonstrated in the in vivo conditions. Two hundred milligrams racemic INDB tablets or intramuscular injections were administered to healthy volunteers. Blood samples (5 ml) were obtained (in serum Gel tubes S/4.7 ml, Sarstedt Monovette, Germany) from the subjects right antecubital fossa at the following times: immediately before administration of INDB and 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 9.0, 12 and 24 h after administration. Within 30 min following blood withdrawal, the samples were centrifuged. The separated serum samples were frozen in plastic vials at 20°C until analysed. The serum concentration INDB enantiomers were used to calculate the following parameters: elimination rate constant (K, h^{-1}), half-life time ($t_{0.5}$, h), area under the serum concentration curve

Table 1
Recovery of indobufen enantiomers from human serum[a]

Nominal indobufen enantiomers concentration (μg ml^{-1})	% Recovery ± SEM	
	(−)*R*-Indobufen	(+)*S*-Indobufen
1.0	94.3 ± 2.0	92.1 ± 1.8
10.0	93.7 ± 1.4	94.1 ± 1.6

[a] $n = 5$

(AUC$_{0\rightarrow\infty}$, μg h ml^{-1}), time peak serum concentration ($t_{max,}$ h), peak serum concentration (C_{max}, μg ml^{-1}). TOPFIT 2.0 software (Gustaw Fisher, Stuttgart, 1993) was used for calculation of the above pharmacokinetic parameters from data human serum concentrations.

2.8. Statistical analysis

Mean concentrations and pharmacokinetic parameters of the enantiomers were analysed using one side ANOVA-test. The standard error mean (SEM) was used to express the tendency of the data. Comparison of parameters with probabilities (P) of no difference $0.001 \leq P < 0.01$ are termed very significantly different (VS). When $0.01 \leq P < 0.05$, the parameters are termed significantly (S) different. Values $P > 0.05$ do not permit rejection of null hypothesis of no differences and these differences are considered to be nonsignificant.

3. Results and discussion

3.1. Analytical parameters

3.1.1. Solid phase extraction (SPE), recovery

In the study, the method for estimation of INDB enantiomers in human serum has been described. For INDB enantiomers isolation from human serum solid phase extraction has been employed, using C18 phase, chemically bound to silica gel instead of classic liquid–liquid extraction using ethyl ether [4]. The latter compound is much more volatile and, therefore, more toxic than methanol at room temperature, at which the studies have been performed. INDB extraction from serum had to be preceded by acidification with 0.1 M l^{-1} orthophosphoric acid. Methanol has proved to represent an appropriate reagent for elution of INDB enantiomers since even its small volume, like 0.3 ml, has been sufficient to obtain the effect. The process of the analysed compound extraction has been quite efficient, ranging from 92.1 to 94.3% (Table 1).

3.1.2. Column selection

The enantiomer separation has been attempted to be achieved by a direct technique, using columns with a chiral phase. At first, Whelk O1 column was used, with the normal phase set-up, in which (3S, 4S)-4-(3, 5-dinitrobenzamido)-1, 2, 3, 4-tetrahydrofenantrene was used as the solid chiral phase. The column was successfully used for separation of 2-aryl propionic acid (2-APA) derivatives: ibuprofen and flurbiprofen, extracted earlier from human serum [10]. In the case of INDB enantiomers, i.e. compounds structurally related to 2-APA derivatives, the separation has proved to be insufficiently selective and this has been so after a relatively long retention time, which is in practice not feasible has been inconvenient. Introduction of a heterocyclic indolyl group transforms this butanoic acid derivative into a substance slightly soluble in hexane, in contrast to ibuprofen or flurbiprofen, the enantiomers of which are very selectively and rapidly separated in the Whelk O1 column. Perhaps, this might have been the principal reason of the poor enantiomer separation in the Whelk O1 column. Application of the Chiradex column with a reversed phase set-up, with the chiral selector in the form of β-cyclodextrine system has also failed to provide adequate separation. Therefore, the achiral C_{18} column (Purospher) was employed, with the reversed phase set-up. INDB enantiomers were derivatised using L-leucinamide in the medium of ethyl chloroformate and triethylamine and separated at relatively short retention times of 3.5 min for (−)*R*-enantiomer and 4.7 min for (+)*S*-enantiomer.

3.1.3. Internal standard

Racemic form of FBP has been used as an internal standard since it has represented the most suitable compound among the analysed substances. Naproxen, piroxicam, ketoprofen, mefenamic acid and indolylacetic acid have manifested retention times so similar to INDB enantiomers that their peaks could not have been separated which, in practice, has eliminated them as internal standards. Retention times of FBP enantiomers have been by a few minutes longer than those of INDB enantiomers, which has permitted to analyse a single sample within 15 min, including elution of endogenous compounds and establishing of the baseline, free of additional peaks (Fig. 2). It should be stressed that, in contrast to the analysed indomethacin or gliclazide, INDB has not been decomposed in the alkaline medium, in which the derivatising of INDB enantiomers took place.

3.1.4. Selectivity

The separated peaks of INDB enantiomers and of the IS are presented in Fig. 2. No peaks interfering with INDB enantiomer or FBP peaks have been detected, which could originate from endogenous compounds in the serum. This has indicated appropriate selectivity of the elaborated procedure.

3.1.5. Linearity, standard curve

The standard curve has been prepared for the range of concentrations from 0.25 to 25.00 μg ml^{-1} for each INDB enantiomer. The range has covered enantiomer levels which could be expected following the administration of a single dose of 200 mg racemic INDB. It should be borne in mind that exceptionally low serum volumes (0.2 ml) have been taken for the estimations, much lower than 1 ml serum quoted in the literature [4]. The standard curves have been described by equa-

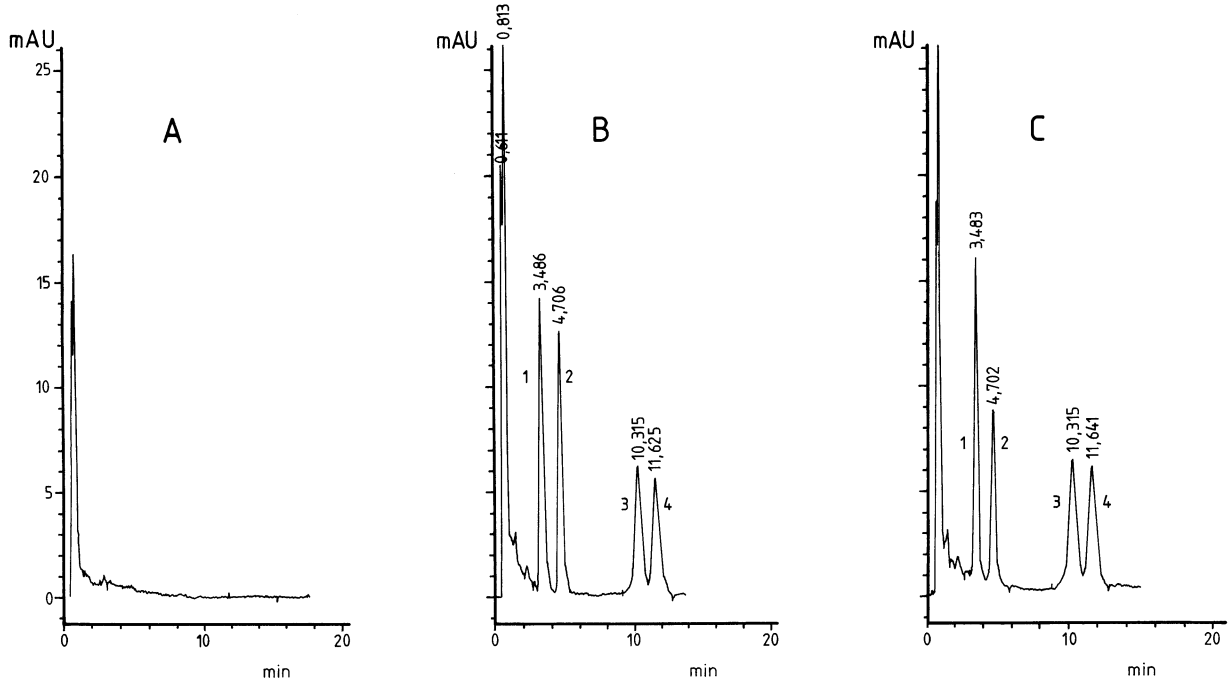

Fig. 2. HPLC chromatograms of indobufen enantiomers human serum previously processed according to the worked out SPE procedure; A, blank serum, B, the blank serum spiked with 5 μg ml^{-1} of each (−)*R*- and (+)*S*-INDB, C-serum sample of volunteer at 6 h elapsed from administration of single dose 200 mg racemic INDB [5.9 μg ml^{-1} (−)*R*-and 3.5 μg ml^{-1} (+)*S*-INDB]. Racemic FBP in serum sample was equal 25.0 μg ml^{-1}. (1) and (2) correspond to (−)*R*- and (+)*S*-INDB, respectively, and (3) and (4) denote FBP enantiomers.

Table 2
Intra- and inter-day precision and accuracy data of standard curves for the analysis of (−)*R*- and (+)*S*-INDB enantiomers in human serum[a]

Nominal concentration (μg ml^{-1})	(−)*R*-INDB		(+)*S*-INDB	
	Assayed value (μg ml^{-1})	CV (%)	Assayed value (μg ml^{-1})	CV (%)
Inter-day repeatability ($n=5$)				
0.25	0.24	8.7	0.23	9.3
0.50	0.51	6.5	0.55	7.1
1.00	1.08	2.2	1.08	6.4
2.50	2.38	3.9	2.61	5.2
5.00	5.20	5.3	5.12	4.8
10.00	10.00	3.0	9.92	3.3
20.00	20.10	3.5	19.70	3.0
25.00	25.90	3.2	26.00	3.9
linear equation $y_1=(0.100\pm0.001)x+0.04$ $r_1=0.9997$			$y_2=(0.099\pm0.001)x+0.03$ $r_2=0.9998$	
Inter-day reproducibility ($n=5$)				
0.25	0.27	9.8	0.23	5.4
0.50	0.53	8.8	0.55	5.9
1.00	1.08	2.6	1.05	5.1
2.50	2.48	3.4	2.57	6.9
5.00	5.06	7.3	5.00	9.1
10.00	10.50	3.1	10.00	4.6
20.00	20.20	3.2	20.00	3.0
25.00	24.70	2.4	24.90	2.5

[a] r is the correlation coefficient.

tions presented in Table 2. The value b at which the standard curve intersected the ordinate (statistically not different from 0) has pointed to the absence of additional peaks in the vicinity of peaks originating from INDB enantiomers.

3.1.6. Precision and accuracy

The intra-day and inter-day accuracies have been estimated and for all studied concentrations of the standard curve have been lower than 10%, as indicated by the respective values of (CV) (Table 2). This has indicated that the method is quite precise. Moreover, the small differences ($\leq$ 10%) noted between nominal levels and the estimated concentrations have documented an appropriate accuracy of the elaborated method.

3.1.7. Limit of detection and quantification

The limit of detection has been 0.1 μg ml^{-1} at the signal to noise ratio of 4:1. The of LoQ in serum, defined as the lowest concentration on the standard curve for which the assay precision has been reflected by CV $\leq$ 10%, has amounted to 0.25 μg ml^{-1} for each of the INDB enantiomers.

3.1.8. Application of the method for the in vivo studies

The worked out method has been successfully applied to quantification of INDB enantiomers after oral or intramuscular administration of 200 mg racemic INDB in each of 20 healthy volunteers and collection of 400 serum samples. The data on INDB (−)*R*- and (+)*S*-enantiomer concentrations in man and their pharmacokinetic parameters in 10 healthy volunteers following intramuscular administration of 200 mg rac-INDB are shown in Table 3. After intramuscular injections of rac-INDB absorption of its enantiomers is faster ($t_{max(-)\text{-R}}=0.9\pm0.2$ h; $t_{max(+)\text{-S}}=0.7\pm0.1$ h if compared with oral suspension administration, $t_{max(-)\text{-R}}=1.87\pm0.33$ h; $t_{max(+)\text{-S}}=1.50\pm0.28$ h) [4]. In both routes of

Table 3
Serum concentration (mean ± SEM) of (−)*R* and (+)*S*-indobufen versus time and pharmacokinetic parameters[a,b]

Time (h)	Concentration (mean ± SEM) (μg ml^{-1})		ANOVA test
	(−)*R*-enantiomers	(+)*S*-enantiomers	
0.5	11.08 ± 0.73	9.86 ± 0.61	NS
1.0	12.52 ± 0.94	10.71 ± 0.77	NS
2.0	10.68 ± 0.80	8.41 ± 0.63	S
3.0	9.91 ± 0.77	6.99 ± 0.56	S
4.0	7.91 ± 0.65	5.45 ± 0.42	VS
6.0	5.73 ± 0.45	3.67 ± 0.35	VS
9.0	3.78 ± 0.37	2.05 ± 0.20	VS
12.0	2.86 ± 0.33	1.48 ± 0.16	VS
24.0	1.10 ± 0.24	0.51 ± 0.08	S
AUC (μg h ml^{-1})	95.5 ± 10.3	59.0 ± 5.7	VS
C_{max} (μg ml^{-1})	13.1 ± 0.7	11.4 ± 0.6	NS
t_{max} (h)	0.9 ± 0.2	0.7 ± 0.1	NS
$t_{0.5}$ (h)	4.7 ± 0.6	3.3 ± 0.4	S
Cl (l/h)	1.2 ± 0.2	1.8 ± 0.3	S
MRT (h)	7.1 ± 0.8	5.2 ± 0.5	S

[a] From 10 healthy volunteers after administration of 200 mg racemic indobufen by intramuscular injection (Jelfa, S.A.)
[b] $AUC_{0\rightarrow\infty}$,the area under curve concentration-time; C_{max},maximum concentration at t_{max}; $t_{0.5}$,elimination half life time; Cl, serum drug clearance; MRT, mean residence time; ANOVA-test, $\alpha = 0.05$, differences between (−)*R*- and (+)*S*- of the indobufen: NS, non significantly; VS, very significantly ($0.001 \leq P < 0.01$); S, significantly ($0.01 \leq P < 0.05$)

administration (−)*R*-enantiomer is slower eliminated than its antipode (e.g. in the case of intramuscular injection $t_{0.5(-)\text{-R}} = 4.7 \pm 0.6$ h, $t_{0.5(+)\text{-S}} = 3.3 \pm 0.4$ h.

4. Conclusion

Application of solid phase extraction has permitted to obtain a relatively high recovery of extraction (over 90%) for each of the analysed INDB enantiomers and has allowed to eliminate the more volatile and, thus, more toxic ethyl ether. The requirement of the low serum volume (0.2 ml) in assays of INDB enantiomers should be stressed. The designed procedure fulfils the validation requirements, is repeatable and reproducible, adequately accurate and precise and can be applied for the in vivo studies. The method has been successfully used in studies on bioavailability of INDB enantiomers following their administration in tablets or in intramuscular injections to healthy individuals. The investigations have been approved by the Human Investigation Ethical Committee at the University of Medical Sciences in Poznan.

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