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# Validation of a high-performance liquid chromatographic method for the determination of ibuprofen enantiomers in plasma of broiler chickens

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**Abstract**

To characterise the pharmacokinetic properties of each enantiomer of ibuprofen in broiler chickens, a stereospecific HPLC method based on a $\alpha_1$-acid glycoprotein bonded chiral stationary phase has been validated. *S*-(+)-naproxen was used as internal standard. Enantiomers of ibuprofen and *S*-(+)-naproxen were baseline separated using a mobile phase consisting of 0.1 *M* phosphate buffer pH=7 and 0.4% 2-propanol. The method is precise, specific, accurate and reproducible. Recoveries were higher than 80% and the limits of quantification for *R*-(−)- and *S*-(+)-ibuprofen were 1.16 and 1.37 μg $ml^{-1}$, respectively. The method seemed suitable for the pharmacokinetic studies of ibuprofen in chickens. 

*Keywords:* Enantiomeric separation; Ibuprofen

## 1. Introduction

Chiral non-steroidal anti-inflammatory drugs (NSAIDs) as ibuprofen are used in food producing animals e.g. the poultry industry. Ibuprofen reduces symptoms of heat stress and clinical signs of coccidiosis and provides an adequate feed consumption and a body weight gain [1]. Ibuprofen is marketed as a racemate. It is pointed out that in many pharmacologically active chiral compounds the two optical isomers have widely different biological effects [2]. In the case of ibuprofen, there seems not to be a compelling case for restricting to the active *S*-(+)-ibuprofen as the racemate has been in use for two decades without serious problems, and no adverse effects have been convincingly attributed to the *R*-(−)-isomer. In vitro, *S*-(+)-ibuprofen exhibits pharmacological effects by inhibition of cyclo-oxygenase (COX), while *R*-(−)-ibuprofen seems to be inactive [3]. In vivo, the pharmacological effects are mainly due to the *S*-(+)-enantiomer and besides, *R*-(−)-ibuprofen is partially converted to *S*-(+)-ibuprofen. In general, in humans and most other species this inversion seems to be unidirectional, while for some species a bidirectional inversion is described [4,5]. For broiler chickens, no data are available. Different

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HPLC-assays for the separation of ibuprofen enantiomers in human plasma have been described. Indirect methods by formation of diastereomeric derivatives of the racemic molecule followed by separation on a non-chiral column have been described [6–8]. Direct methods for the separation of ibuprofen enantiomers are based on chiral mobile phases or chiral stationary phases (CSPs). Pettersson et al. determined the enantiomers of ibuprofen in plasma using an $\alpha_1$-acid glycoprotein (AGP) bonded chiral stationary phase [9]. Devries et al. also analysed ibuprofen enantiomers in human plasma and urine by using an $\alpha_1$-acid glycoprotein (AGP) bonded chiral stationary phase [10]. Andrisano et al. described the enantiomeric separation of chiral arylcarboxylic acids as ibuprofen on an immobilized human serum albumin chiral stationary phase [11]. Tang [12] and Van Overbeke et al. [13] described the separation of ibuprofen enantiomers on a cellulose based chiral stationary phase. Geisslinger et al. determined ibuprofen enantiomers in biological fluids by using a β-cyclodextrine stationary phase [14].

No data on the pharmacokinetics of the enantiomers of ibuprofen in broiler chickens are available. A method for the quantification of the enantiomers of ibuprofen in plasma of broiler chickens was developed and validated for the pharmacokinetic evaluation of both enantiomers.

## 2. Experimental

### 2.1. Chemicals

Racemic ibuprofen (Knoll Pharma Chemicals, Nottingham, UK) and *S*-(+)-naproxen (Fluka, Neu-Ulm, Germany), used as the internal standard, were of pharmaceutical and analytical grade, respectively. Sodium dihyrogenphosphate dihydrate (Vel, Leuven, Belgium), 2-propanol (Lab Chemistry, Novolab, Geraardsbergen, Belgium) and tetrahydrofuran (Labscan, Dublin, Ireland) were of HPLC grade. Sodium hydroxide (Acros, Geel, Belgium), hydrochloric acid 37% (Vel) and hexane (Vel) were of analytical grade. The HPLC solvents were degassed before use.

### 2.2. Instrumentation and chromatographic conditions

The HPLC system consisted of an isocratic HPLC pump (L-6000, Merck-Hitachi, Tokyo, Japan), a septumless syringe-loaded injector (Valco six-channel injector, Valco Instruments, Houston, TX, USA) equipped with a loop of 25 μl (Valco Instruments Corp.), a Chiral–AGP® column (100×4 mm) (Chromtech AB, Norsborg, Sweden), a Chiral-AGP® guard column (10×3 mm) (Chromtech AB), a variable UV-detector (L-4200, Merck-Hitachi) set at 220 nm and an integrator (D-2000, Merck-Hitachi). The analysis was performed at ambient temperature and the flow-rate was set at 0.9 ml $min^{-1}$. The mobile phase consisted of 0.4% (v/v) 2-propanol in a 0.1 *M* phosphate buffer pH 7. For the preparation of the calibration curves, a stock solution of ibuprofen (500 μg $ml^{-1}$) and different working solutions (1.56, 3.125, 6.25, 12.5, 25 and 50 μg $ml^{-1}$) were prepared in ethanol. An internal standard solution containing 25 μg $ml^{-1}$ *S*-(+)-naproxen was prepared in ethanol. Fifty μl of an ibuprofen solution and 50 μl of the internal standard solution were added to 500 μl blank plasma and transferred into 16×100 mm tubes (Pyrex® Disposable Culture Tubes, Corning, New York, USA). This mixture was vortexed for 3 s and then 200 μl 1 *M* HCl and 5 ml hexane–2-propanol (v/v; 95:5) were added. After 1 min vortexing and 5 min centrifugation at 1000 *g*, 4.5 ml of the organic layer was transferred to a clean tube and evaporated to dryness at 40°C under a stream of nitrogen. The residues were dissolved in 250 μl of mobile phase and injected onto the HPLC system.

### 2.3. Influence of the pH of the mobile phase and the addition of uncharged organic compounds as 2-propanol to the mobile phase on the enantioselectivity

To study the influence of the pH of a 0.1 *M* phosphate buffer mobile phase on the retention times and enantioselectivity of the enantiomers of ibuprofen, mobile phases with a pH varying from 5.5 to 7 were prepared. Spiked plasma samples of ibuprofen (10 μg $ml^{-1}$) and *S*-(+)-naproxen (25 μg

$ml^{-1}$) were extracted and analysed as described before.

To study the influence of the addition of uncharged organic compounds on the retention times and enantioselectivity of the enantiomers of ibuprofen, mobile phases with a different concentration of 2-propanol ranging from 2 to 0.4% were prepared. Spiked plasma samples of ibuprofen (10 μg $ml^{-1}$) and *S*-(+)-naproxen (25 μg $ml^{-1}$) were extracted and analysed as described before.

### 2.4. Validation

Specificity was determined at 220 nm by using a diode-array detector to compare the chromatograms after extraction of blank plasma and of plasma spiked with racemic ibuprofen (10 μg $ml^{-1}$) and *S*-(+)-naproxen (25 μg $ml^{-1}$). Linearity was tested by extraction and injection of 9 calibration curves. For each calibration curve, plasma was spiked with various amounts of ibuprofen to obtain concentrations from 0 to 50 μg $ml^{-1}$. Precision was calculated at concentrations of 1.56, 6.25, 25 and 50 μg $ml^{-1}$ by the interpretation of the within-day variation (repeatability) and between-day variations (reproducibility). The repeatability was determined by comparison of the variations of the measured peak areas for the same sample injected at different ($n=5$) times during the same day. For the reproducibility, the samples were injected at five different days. The precision around the mean value should not exceed 15% coefficient of variation [15]. The values (1.56, 6.25, 25 and 50 μg $ml^{-1}$) used for the determination of the accuracy were determined by applying the test procedure 10 times. The mean value should be within ±15% of the actual value [15]. Recoveries were evaluated by comparing extracted spiked samples with non-extracted standard solutions in mobile phase. The recovery values were performed at ibuprofen concentrations of 1.56, 6.25, 25 and 50 μg $ml^{-1}$. The limit of detection is the concentration of the analyte which gives a measured signal equal to the blank signal plus three times the standard deviation of the blank [16]. The concentration of the analyte which gives a measured signal equal to the mean blank signal plus ten times the standard deviation of the blank is expressed as the limit of quantification. The blank signal was defined as the average of the *y*-intercept of 9 calibration curves in plasma [16].

### 2.5. Pharmacokinetic study of ibuprofen in broiler chickens

#### 2.5.1. Animals

8 male sex Ross chickens with a weight of 3±0.5 kg were used. They were preconditioned for a week at a temperature of 20±2°C with a 12 h dark–12 h light period. The chickens were permitted free access to feed and water but 24 h before the experiment, feed was removed while water was available ad libitum.

#### 2.5.2. Intravenous administration of ibuprofen

At the age of 6 weeks, the chickens were administered intravenously into the wing vein a dose equivalent to 15 mg ibuprofen per kg body weight. The intravenous solution was prepared by dissolving 10 g ibuprofen in 40 g absolute ethanol (Merck, Darmstadt, Germany). Next, this solution was diluted with water for injection (Federa, Brussels, Belgium) to a weight of 100 g after addition of 2 *N* sodium hydroxide to a pH of 7.4. After filtration through a sterile filter (0.22 μm, Schleicher and Schuell, Dassel, Germany), a sterile syringe (1 ml, 26 G $\frac{1}{2}$, 0.45×13, Becton Dickinson, Madrid, Spain) was filled in function of the weight of the chickens. Blood samples were collected from the tight vein at different time points (0, 2, 5, 10, 30, 45, 60, 120 and 240 min) in tubes containing heparin (Heparin Leo, Leo Pharmaceuticals Products, Zaventem, Belgium) as anticoagulant. Samples were centrifuged (1000 *g*) for 10 min and stored at −20°C until analysis. After addition of 50 μl internal standard solution *S*-(+)-naproxen (25 μg $ml^{-1}$) to 500 μl plasma, the samples were extracted, re-dissolved and injected as described before.

#### 2.5.3. Oral bolus administration of ibuprofen into the crop

At the age of 8 weeks, the chickens used for the intravenous injection, received a bolus into the crop of a commercially available ibuprofen suspension (Junifen®, Boots, Groot-Bijgaarden, Belgium) in a

dose of 15 mg ibuprofen per kg body weight. The administration was performed with a flexible plastic tubing system (Ext Line 15 LLM–LLF, 1.5×4.1, Beldico, Aye, Belgium) intubated into the crop. Blood samples were collected from the tight vein at different time points (0, 10, 20, 30, 45, 60, 75, 90, 120, 180 and 240 min) in tubes containing heparin as anticoagulant. Next, the samples were treated as described above.

#### *2.5.4. Data analysis*

The calibration curves were calculated by linear regression of the peak-area ratio versus concentration. The individual plasma concentration time profiles were calculated using the MW-PHARM program version 3.0 (Mediware 1987–1991, Utrecht, The Netherlands). The $AUC_{0-4\ h}$ values ($\mu g\ h\ ml^{-1}$) were calculated using the logarithmic and linear trapezoidal rules.

## 3. Results and discussion

### *3.1. Influence of pH of the mobile phase and 2-propanol added to the mobile phase on the enantioselectivity of ibuprofen enantiomers*

The enantioselectivity increased by modifying the pH of the mobile phase from 5.5 to 7 and can be explained by a change of the conformation of AGP and an excessive retention of non-ionised enantiomers to AGP inducing an inadequate separation at a lower pH. While the pH of the mobile phase increased, the retention of the enantiomers decreased because AGP (IEP 2.7) and the enantiomers of ibuprofen were both negatively charged ($pK_a$ 4.4–5.2) at this pH value. Fig. 1a shows a chromatogram of the enantiomers of ibuprofen (10 $\mu g\ ml^{-1}$) and *S*-(+)-naproxen (25 $\mu g\ ml^{-1}$) at pH 5.5 while Fig. 1b shows a chromatogram of the same compounds at pH 7. At a pH value of 5.5, the retention times of the enantiomers were 13.26 min and 17.53 min for *R*-(−) and *S*-(+)-ibuprofen, respectively while the retention time of *S*-(+)-naproxen was 23.47 min (a). At a pH value of 7, the retention times were 3.86 min and 5.13 min for *R*-(−) and *S*-(+)-ibuprofen, respectively. The retention time of *S*-(+)-naproxen was 6.76 min (b).

As referred in literature, different uncharged organic compounds as tetrahydrofuran, acetonitrile and 2-propanol can be used [17]. In our experiments, a complete separation of the enantiomers was obtained at a 2-propanol concentration of 0.4% (v/v) added to a 0.1 *M* phosphate buffer pH 7. As described in literature, retention and enantioselectivity decreased by increasing the concentration of 2-propanol [17].

### *3.2. Validation*

The chromatograms shown in Fig. 2 indicated that there was no interference of endogenous compounds and the anticoagulant with the ibuprofen enantiomers and *S*-(+)-naproxen.

The mean ($n=9$) calibration curve coefficients for *R*-(−)-ibuprofen and *S*-(+)-ibuprofen are listed in Table 1. The calibration curves were linear over the entire concentration range (0–50 $\mu g\ ml^{-1}$). For repeatability and reproducibility, the coefficients of variation (C.V.) are shown in Table 2. The coefficients of variation for the within-day variation and between-day variations were all lower than 10%.

The proposed method can be considered accurate as for the concentrations of 1.56, 6.25, 25 and 50 $\mu g\ ml^{-1}$ by applying the test procedure 10 times, coefficients of variation lower than 15% were obtained as shown in Table 3.

The percentage recovery from chicken plasma was 82±2%, for *R*-(−)-ibuprofen and 85±1% for *S*-(+)-ibuprofen at concentrations of 1.56, 6.25, 25 and 50 $\mu g\ ml^{-1}$. For *S*-(+)-naproxen (25 $\mu g\ ml^{-1}$), a recovery of 87±2% was obtained.

Considering the values of the mean regression equation of *R*-(−)-ibuprofen and *S*-(+)-ibuprofen as shown in Table 1, the limit of detection and the limit of quantification for *R*-(−)-ibuprofen was 0.35 and 1.16 $\mu g/ml$, respectively. For *S*-(+)-ibuprofen, the limit of detection and limit of quantification was 0.41 and 1.37 $\mu g/ml$, respectively.

We can conclude that the proposed method for the determination of the ibuprofen enantiomers in plasma of broiler chickens, is specific, accurate, repeatable and reproducible. Linear calibration curves ($r^2>0.999$) were obtained in plasma between 0 and 50 $\mu g\ ml^{-1}$ for *R*-(−)-ibuprofen and *S*-(+)-ibuprofen. For the recoveries, values above 80% were obtained.

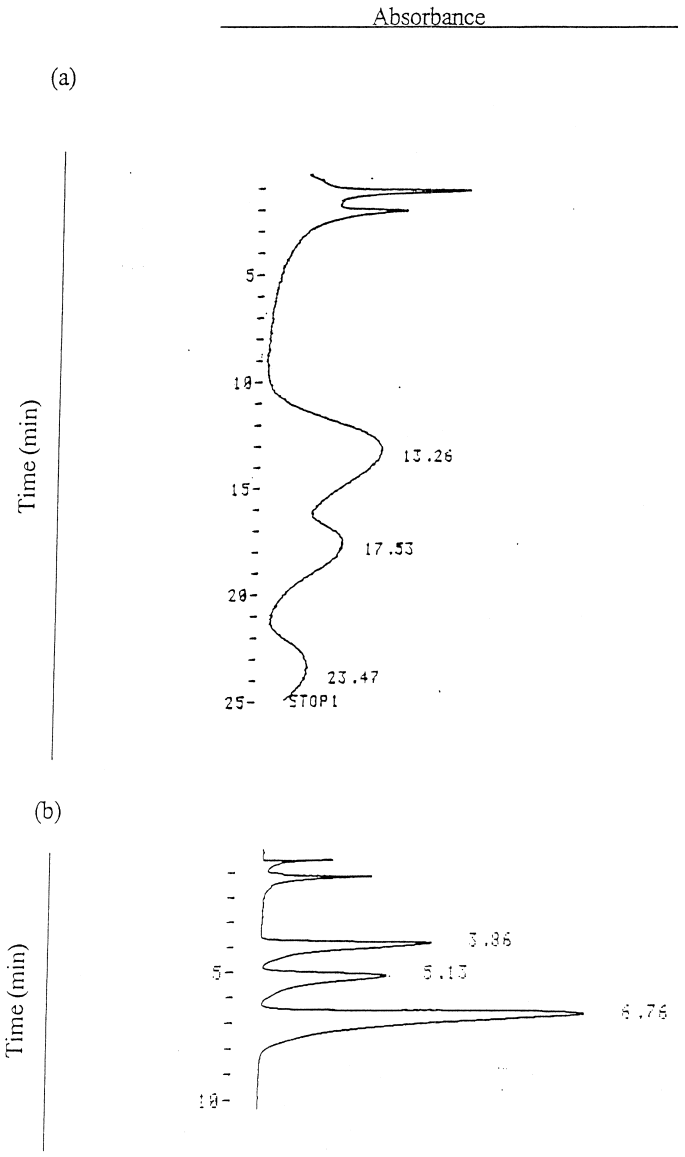


Fig. 1. Influence of pH on the enantioselectivity of the enantiomers of ibuprofen (a) pH = 5.5. Retention times of 13.26, 17.53 and 23.47 min for *R*-(−)-ibuprofen, *S*-(+)-ibuprofen and *S*-(+)-naproxen, respectively. (b) pH = 7. Retention times of 3.86, 5.13 and 6.67 min for *R*-(−)-ibuprofen, *S*-(+)-ibuprofen and *S*-(+)-naproxen, respectively.

### *3.3. Pharmacokinetics of ibuprofen in broiler chickens*

Fig. 3a–b show the mean plasma concentration time profiles after intravenous and oral administration of a dose of 15 mg ibuprofen per kg body weight to 8 chickens for *R*-(−)-ibuprofen (a) and *S*-(+)-ibuprofen (b), respectively. Fig. 4 shows the individual plasma concentration time profiles of *S*-(+)-ibuprofen after oral bolus administration of 15 mg per kg body weight to 8 chickens. A large variability in individual plasma concentration time

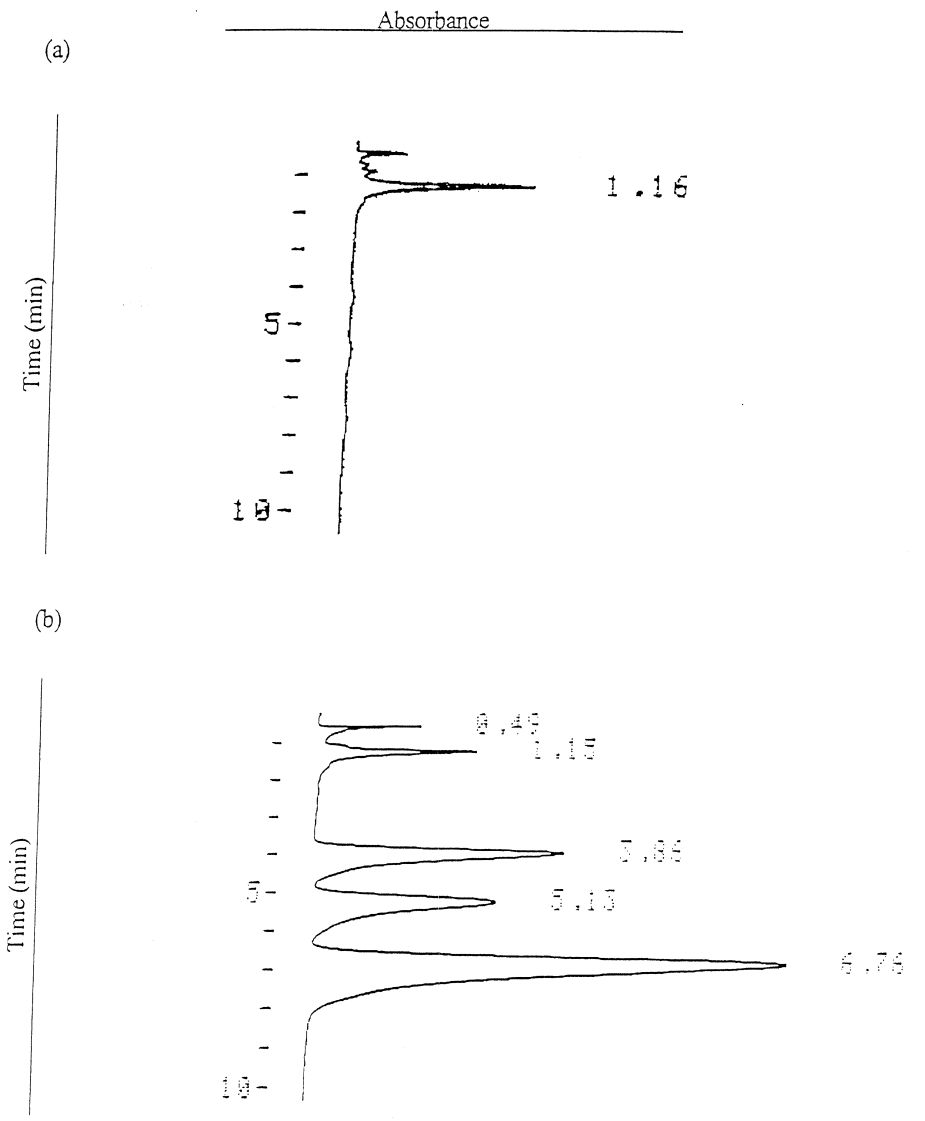


Fig. 2. Chromatograms after extraction of blank chicken plasma (a) and after extraction of plasma containing 10 μg $ml^{-1}$ racemic ibuprofen and 25 μg $ml^{-1}$ *S*-(−)-naproxen (b).

profiles was observed. Some profiles show a multiple peaking. These multiple peaks can be caused by a gradual absorption process or an absorption window. No data about the absorption mechanism of NSAIDs as ibuprofen in chickens are available. Because of the fact that multiple peaking has also occurred after intravenous administration of ibuprofen, other reasons as absorption mechanisms are responsible for this phenomenon. Double peaking can also be explained by the occurrence of an enterohepatic circulation. Enterohepatic circulation was reported for different non-steroidal anti-inflammatory drugs in different species. Indomethacin [18], piroxicam [19], diclofenac [18] and sulindac [20] have been reported

Table 1
The mean±SD ($n=9$) calibration curve for *R*-(−)-ibuprofen and *S*-(+)-ibuprofen expressed by a coefficient of correlation and a regression equation

| Concentration ($\mu g\ ml^{-1}$) | *R*-(−)-ibuprofen | *S*-(+)-ibuprofen |
|---|---|---|
| 0 | 0±0 | 0±0 |
| 1.56 | 0.060±0.007 | 0.078±0.004 |
| 3.125 | 0.153±0.006 | 0.188±0.006 |
| 6.25 | 0.368±0.014 | 0.430±0.013 |
| 12.5 | 0.792±0.023 | 0.868±0.028 |
| 25 | 1.619±0.054 | 1.762±0.086 |
| 50 | 3.042±0.052 | 3.359±0.111 |
| Intercept | −0.0075±0.006 | 0.0004±0.0077 |
| Slope | 0.0617±0.0011 | 0.0679±0.0024 |
| Correlation | 0.9992±0.0005 | 0.9996±0.0003 |
| Regression equation | $y=0.0618x-0.0075$ | $y=0.0679x+0.0004$ |

Table 2
Coefficients of variation (C.V.) (%) ($n=5$) for the precision by within-day variation (repeatability) and between-days variation (reproducibility)

| Concentration ($\mu g\ ml^{-1}$) | *R*-(−)-ibuprofen (C.V.%) | *S*-(+)-ibuprofen (C.V.%) |
|---|---|---|
| *Repeatability* | | |
| 1.56 | 9.4 | 3.4 |
| 6.25 | 3.7 | 2.2 |
| 25 | 2.8 | 3.2 |
| 50 | 2.1 | 2.9 |
| *Reproducibility* | | |
| 1.56 | 5.7 | 3.9 |
| 6.25 | 2.6 | 2.4 |
| 25 | 3.1 | 2.5 |
| 50 | 0.9 | 1.6 |

to show an enterohepatic recirculation in humans. Piroxicam undergoes enterohepatic recirculation in beagle dogs [19] and rabbits [21]. In rats, an enterohepatic recirculation occurred after the administration of ibuprofen [22]. For chickens, no reports about the occurrence of an enterohepatic circulation after administration of NSAIDs are available. Due to the supposed occurrence of an enterohepatic recirculation, area under the curve (AUC) values were not used to calculate the bioavailability and other pharmacokinetic parameters based on AUC values. AUC values after intravenous administration and oral bolus administration of 15 mg ibuprofen per kg body weight are listed in Table 4. The difference in AUC values of *R*-(−)-ibuprofen and *S*-(+)-ibuprofen after the administration of racemic ibuprofen allows to suggest that an inversion from *R*-(−)-ibuprofen to *S*-(+)-ibuprofen might occur. Because of the fact that the differences in plasma concentration time profiles between *R*-(−)- and *S*-(+)-ibuprofen are already observed 10 min after an oral administration of ibuprofen, it is unlikely that they are originated from the occurrence of a presystemic chiral inversion from *R*-(−)- to *S*-(+)- as described for ibuprofen in humans [23,24] and in dogs [25]. It could be hypothetised that because of stereospecific

Table 3
Calculated values and coefficients of variation (C.V.) (%) ($n=10$) for the accuracy

| Conc. ($\mu g\ ml^{-1}$) | *R*-(−)-ibuprofen | | *S*-(+)-ibuprofen | |
|---|---|---|---|---|
| | Calculated conc. ($\mu g\ ml^{-1}$) | C.V. (%) | Calculated conc. ($\mu g\ ml^{-1}$) | C.V. (%) |
| 1.56 | 1.32 | −12 | 1.35 | −10 |
| 6.25 | 6.13 | −1.9 | 6.32 | 1.26 |
| 25 | 26.2 | 4.8 | 25.71 | 2.86 |
| 50 | 49.4 | −1.2 | 49.4 | −1.2 |

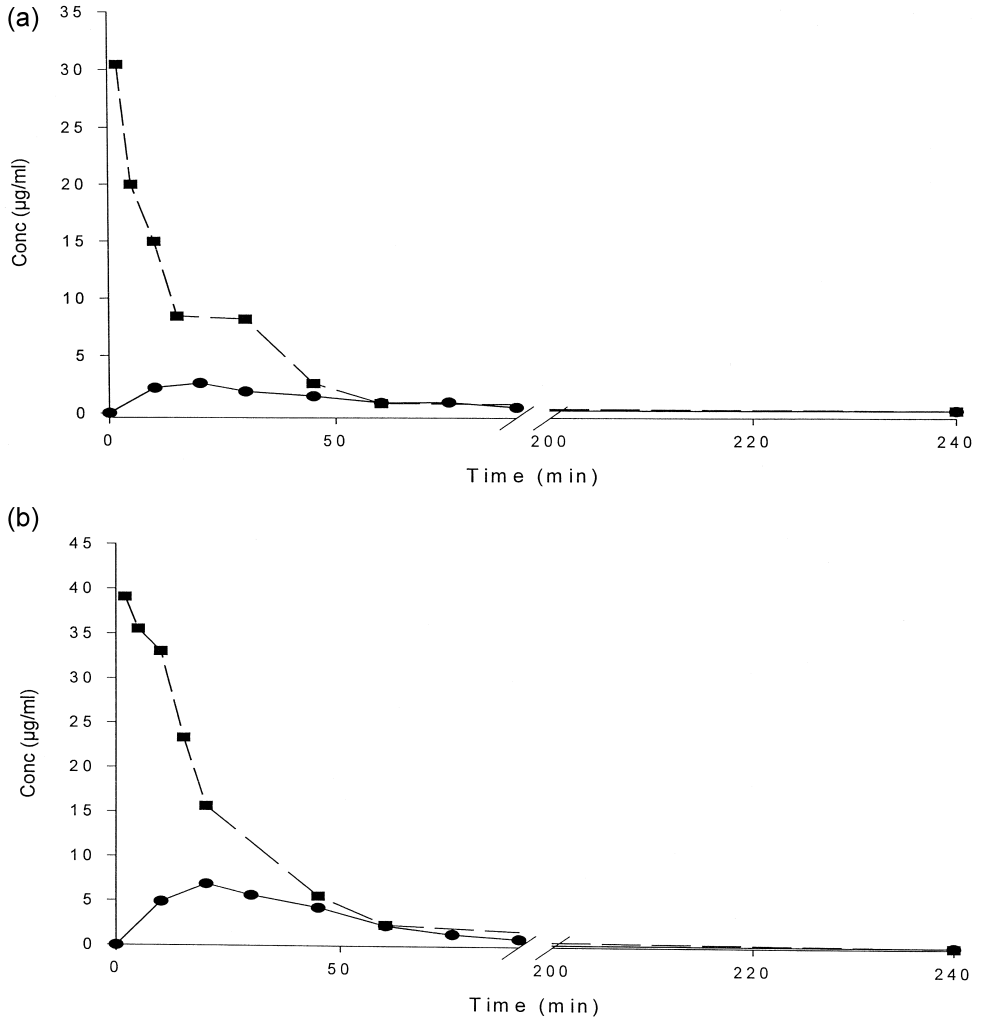


Fig. 3. Mean plasma concentration time profiles of *R*-(−)-ibuprofen (a) and *S*-(+)-ibuprofen (b) after oral bolus administration (●) and intravenous administration (■) of 15 mg ibuprofen/kg body weight to 8 broiler chickens.

absorption mechanisms, both enantiomers are absorbed in a different way. Further on, a significant difference in AUC values between *R*-(−)- and *S*-(+)-ibuprofen has been observed after intravenous administration and this phenomenon also supposed the occurrence of a systemic inversion from *R*-(−)-ibuprofen into *S*-(+)-ibuprofen. More detailed experiments studying the absorption from the gut and the inversion mechanism after administration of *R*-(−)-ibuprofen, *S*-(+)-ibuprofen and the racemate must be undertaken before these hypotheses can be confirmed. Besides, the low AUC-values after an oral bolus administration indicate a low oral bioavailability of ibuprofen in chickens and confirms the data of Roder et al. [1]. However these authors calculated a relative bioavailability value as they did not take the enterohepatic circulation into account.

We can conclude that the method described here allowed the determination of the enantiomers of ibuprofen in plasma of broiler chickens. An enterohepatic circulation of ibuprofen in broiler chickens is described and it can be hypothesised that an unidirectional inversion from *R*-(−)-ibuprofen to *S*-(+)-ibuprofen exists in broiler chickens.

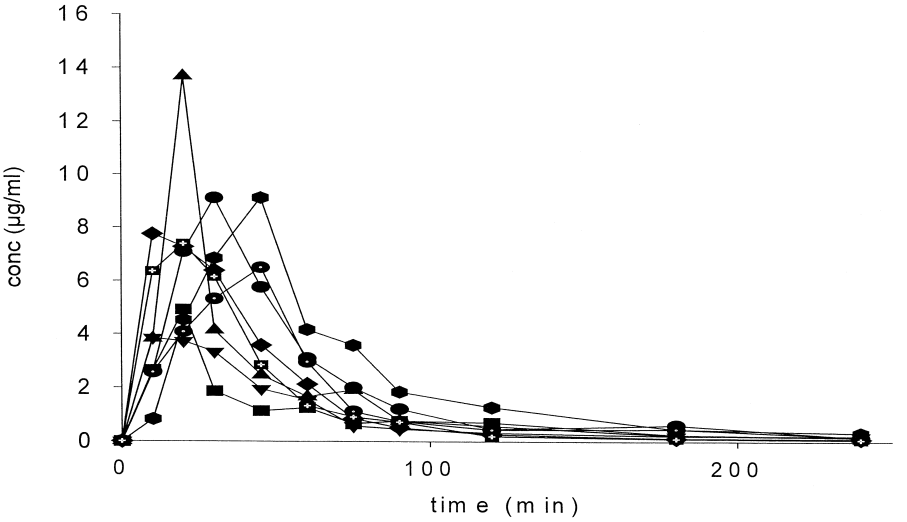


Fig. 4. Individual plasma concentration time profiles of *S*-(+)-ibuprofen after oral bolus administration of 15 mg/kg to 8 broiler chickens.

Table 4
Average AUC values for both ibuprofen enantiomers after intravenous (IV) and oral bolus (OB) administration of 15 mg ibuprofen per kg body weight to broiler chickens ($n=8$)

| Concentration ($\mu g\ ml^{-1}$) | AUC IV ($\mu g\ h/ml$) | AUC OB ($\mu g\ h/ml$) |
|---|---|---|
| *R*-(−)-ibuprofen | 10.59±3.45 | 2.79±1.05 |
| *S*-(+)-ibuprofen | 19.37±5.02 | 5.58±2.14 |

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