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# Quantification of nimesulide in human plasma by high-performance liquid chromatography/tandem mass spectrometry. Application to bioequivalence studies

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**A method based on liquid chromatography with negative ion electrospray ionization and tandem mass spectrometry is described for the determination of nimesulide in human plasma. Liquid–liquid extraction using a mixture of diethyl ether and dichloromethane was employed and celecoxib was used as an internal standard. The chromatographic run time was 4.5 min and the weighted ($1/x$) calibration curve was linear in the range 10.0–2000 ng ml$^{-1}$. The limit of quantification was 10 ng ml$^{-1}$, the intra-batch precision was 6.3, 2.1 and 2.1% and the intra-batch accuracy was 3.2, 0.3 and 0.1% for 30, 300 and 1200 ng ml$^{-1}$ respectively. The inter-batch precision was 2.3, 2.8 and 2.7% and the accuracy was 3.3, 0.3 and 0.1% for 30, 300 and 1200 ng ml$^{-1}$ respectively. This method was employed in a bioequivalence study of one nimesulide drop formulation (nimesulide 50 mg ml$^{-1}$ drop, Medley S/A Indústria Farmacêutica, Brazil) against one standard nimesulide drop formulation (Nisulid, 50 mg ml$^{-1}$ drop, Astra Médica, Brazil). Twenty-four healthy volunteers (both sexes) took part in the study and received a single oral dose of nimesulide (100 mg, equivalent to 2 ml of either formulation) in an open, randomized, two-period crossover way, with a 2-week washout interval between periods. The 90% confidence interval (CI) for geometric mean ratios between nimesulide and Nisulid were 93.1–109.6% for $C_{max}$, 87.7–99.8% for $AUC_{last}$ and 88.1–99.7% for $AUC_{0-\infty}$. Since the 90% CI for the above-mentioned parameters were included in the 80–125% interval proposed by the US Food and Drug Administration, the two formulations were considered bioequivalent in terms of both rate and extent of absorption.** 

**KEYWORDS:** celecoxib; bioavailability; electrospray; pharmacokinetics; biological matrices; high-performance liquid chromatography/tandem mass spectrometry

## INTRODUCTION

Nimesulide (*N*-(4-nitro-2-phenoxyphenyl)methanesulfonamide) is a potent non-steroidal anti-inflammatory drug. It was reported to be a selective inhibitor of cyclo-oxygenase-2 (COX-2) with anti-inflammatory, analgesic and antipyretic properties.[1,2]

A few high-performance liquid chromatographic methods using UV detection have previously been reported for the determination of nimesulide in human plasma.[3,4] To our knowledge, the quantification of nimesulide in human plasma using mass spectrometric detection has not been so far reported.

Because of its higher level of specificity and sensitivity, high-performance liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) is becoming one of the most interesting techniques for the quantification of drugs in biological matrices (such as human plasma).[5,6] Here, we describe a fast, sensitive and specific LC/MS/MS method for the quantification of nimesulide using celecoxib as internal standard (Fig. 1).

This method was applied to a study of the bioequivalence of one nimesulide drop formulation (Nimesulide 50 mg ml$^{-1}$ drop, Medley S/A Indústria Farmacêutica, Brazil) against one standard nimesulide drop formulation (Nisulid 50 mg ml$^{-1}$ drop, Astra Médica, Brazil).

## EXPERIMENTAL

### Materials

HPLC-grade solvents and analytical-reagent grade reagents were purchased from Mallinckrodt (St. Louis, MO, USA) and Nuclear (São Paulo, Brazil). Nimesulide was purchased from Medley S/A Indústria Farmacêutica, Brazil. Celecoxib was a generous gift from Nicox (Nice, France). Blank human blood was collected from healthy, drug-free volunteers. Plasma was

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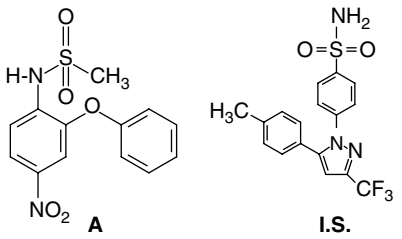


**Figure 1.** Structures of nimesulide (A) and celecoxib (IS).

obtained by centrifugation of blood collected with sodium heparin. Blank plasma was also collected, pooled and stored at ~20 °C until used.

## Calibration standards and quality controls

Nimesulide stock solutions were prepared by sequentially diluting a 1.0 mg ml$^{-1}$ methanol–water (50 : 50, v/v) solution of nimesulide to give standard concentrations in the range 0.2–40 μg ml$^{-1}$. Blank human pooled plasma (28.5 ml) was then spiked with the nimesulide stock solution (1.5 ml) to make standard plasma samples of 10, 20, 50, 100, 200, 500, 1000 and 2000 ng ml$^{-1}$. These standards plasma samples were stored at −20 °C in aliquots until ready to use. Celecoxib was used as an internal standard (IS) at a concentration of 20 μg ml$^{-1}$ in methanol–water (50 : 50, v/v) and stored at 4 °C until used.

The quality control concentrations were fixed at 30, 300 and 1200 ng ml$^{-1}$ (QCA, QCB and QCC, respectively) and were prepared independently of the calibration standards.

## Sample preparation

A 50 μl volume of the IS solution was added to a 200 μl aliquot of each calibration standard or unknown human plasma sample. The tubes were briefly vortex mixed and the compounds of interest were extracted with 4 ml of diethyl ether–dichloromethane (70 : 30, v/v). The mixture was vortex mixed for ~40 s, the upper organic layer was transferred to another set of clean tubes and the organic solvents were evaporated under $N_2$ at 40 °C. The dry residues were reconstituted with 500 μl of mobile phase (see below) and transferred to auto-injector microvials.

## Chromatographic conditions

An aliquot (80 μl) of each plasma extract was injected into an X-Terra TM-MS $C_{18}$ 3.5 μm column (150 × 4.6 mm i.d.) fitted with a guard column of the same material (both column and pre-column temperatures were kept at 40 °C). Chromatography was performed isocratically using a mobile phase of acetonitrile–water (80 : 20) plus 5 mM ammonia solution at a flow-rate of 0.5 ml min$^{-1}$. The pressure of the system was ~50–90 bar with a new guard column in place. The injection volume was 10 μl. Under these conditions, typical standard retention times were 1.3 min for nimesulide and 2.1 min for celecoxib.

A split of the column eluate of ~1 : 10 was included, so that only ~100 μl min$^{-1}$ entered the mass spectrometer. The temperature of the autosampler was kept at 5 °C and the run time was 4.5 min.

## Mass spectrometric conditions

The mass spectrometer (Micromass Quattro II) equipped with an electrospray source was run in the negative mode ($ES^-$), and set up in the multiple reaction mode (MRM), monitoring the transitions $m/z$ 307 → 229 and $m/z$ 380 → 316 for nimesulide and celecoxib, respectively [full-scan spectra are illustrated in Fig. 2(A) and (C)]. For both nimesulide and celecoxib the dwell time was optimized at 0.5 s and the collision energies were 15 and 20 eV, respectively. The collision gas (argon) pressure and the cone voltage were $1.9 \times 10^{-3}$ bar and 31 V, respectively, for for both nimesulide and celecoxib. Data acquisition and analysis were performed using the software MassLynx (v. 3.2) running under Windows NT (v. 4.0) on a Pentium PC.

## Method development

Full-scan negative mass spectra of nimesulide and celecoxib (IS) showed the deprotonated molecules, $[M - H]^-$, of $m/z$ 307 and 380, respectively [Fig. 2(A) and (B)]. The most abundant ion in the product ion spectra was at $m/z$ 229 for nimesulide obtained by loss of 78 u as shown in Fig. 2(C). The ion at $m/z$ 316 was the most abundant product ion for celecoxib (IS) as result of a neutral loss of 64 u ($SO_2$) [Fig. 2(D)]. (for more information on the fragmentation of celecoxib, see Ref. 7). The proposed fragmentation pathways are illustrated in Fig. 3.

From these results, the mass spectrometer was set as follows: $m/z$ 307 for nimesulide and $m/z$ 380 for celecoxib as the precursor ions and $m/z$ 229 and 316 as the respective product ions in the MRM mode. No peak was observed in the mass chromatogram of blank human plasma under the LC/MS/MS conditions previously described, as shown in Fig. 4(A)–(C). The mass chromatograms of a sample are shown in Fig. 4(D)–(F), where it can be observed that the retention times of nimesulide and the IS were 2.36 and 1.14 min, respectively.

## Bioequivalence study

The method was applied to evaluate the bioequivalence of nimesulide in healthy volunteers: nimesulide drops (test formulation from Medley S.A. Indústria Farmacêutica, Brazil; strength 50 mg ml$^{-1}$, batch No. NIG 02/00-1, expiry date 02/2002) and Nisulid (standard reference formulation from Astra Médica, Brazil; strength 50 mg ml$^{-1}$, lot No. 00946, expiry date 08/2003). Bioequivalence between the two formulations was assessed by calculating individual test/reference ratios for the peak of concentration ($C_{max}$), area under the curve (AUC) of plasma concentration until the last concentration observed ($AUC_{last}$) and the area under the curve between the first sample (pre-dosage) and the infinity ($AUC_{inf}$), together with their means and 90% confidence intervals (CI) after logarithmic transformation of the data (additive model). The inclusion of the 90% CI of the ratios in the 80–125% interval was analyzed by ANOVA.

Twenty-four healthy volunteers of both sexes (12 male and 12 female), aged between 18 and 50 years and within 15% of the ideal body weight, were selected for the study after assessment of their health status by clinical evaluation (physical examination, ECG) and the following laboratory

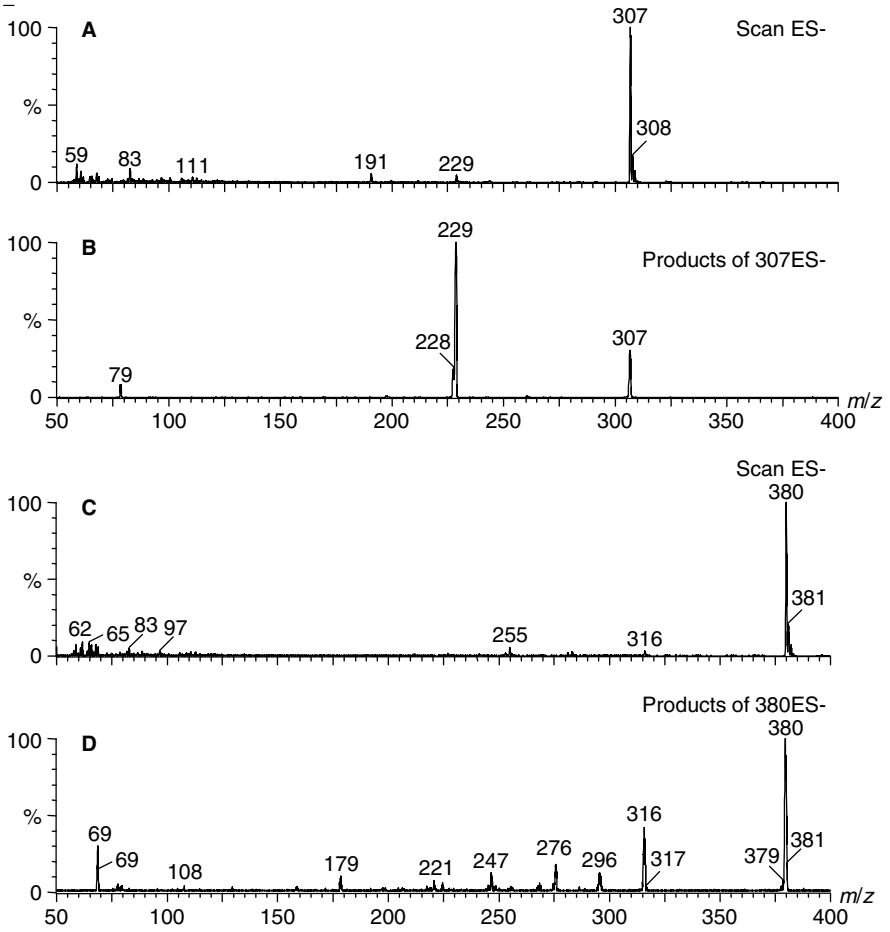


**Figure 2.** Full-scan mass spectra of nimesulide (A), celecoxib (C) and their respective product ion spectra (B and D).

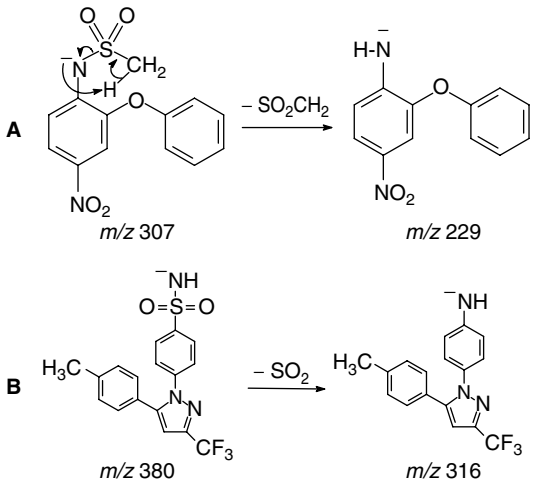


**Figure 3.** Proposed mass fragmentation pathways for nimesulide (A) and celecoxib (B).

tests: blood glucose, urea, creatinine, AST, ALT, alkaline phosphatase, $\gamma$-GT, total bilirubin, albumin and total protein, triglyceride, total cholesterol, hemoglobin, hematocrit, total and differential white cell counts and routine urinalysis. All subjects were negative for HIV, HCV and HBV (except for serological scar).

The volunteers had the following clinical characteristics (divided by gender and expressed as mean $\pm$ SD [range]): male, age $29.6 \pm 6.0$ yr [21–38], height $170.6 \pm 5.1$ cm [161–178], body weight $68.9 \pm 9.3$ kg [60.0–83.0]; female, age $28.6 \pm 5.7$ yr [21–39], height $163.8 \pm 6.8$ cm [155–178], body weight $56.6 \pm 11.4$ kg [36.0–84.0].

The study was a single-dose, two-way randomized crossover design with a 2 week washout period between the doses. During each period, the volunteers were hospitalized at 9:00 p.m. having already had a normal evening meal, and after an overnight fast they received (at 6:00 a.m.) a single dose of nimesulide (100 mg equivalent to a 2 ml suspension volume of either formulation administered by means of a syringe applied directly to the volunteer's mouth). Water (200 ml) was given immediately after the drug administration and the volunteers were then fasted for 4 h, after which period a standard lunch was served; an evening meal was provided 10 h after dosing. No other food was permitted during the 'in-house ' period and liquid consumption was allowed *ad libitum* after lunch (with no xanthine-containing drinks, including tea, coffee and cola).

Systolic and diastolic arterial pressure (measured non-invasively with a sphygmomanometer), heart rate and temperature were recorded just before and hourly after the administration of each nimesulide dose.

Blood samples (8 ml) from a suitable antecubital vein were collected in sodium heparin-containing tubes before and 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 12 and 24 h after dosing. The blood samples were centrifuged at 2000 *g* for 10 min at

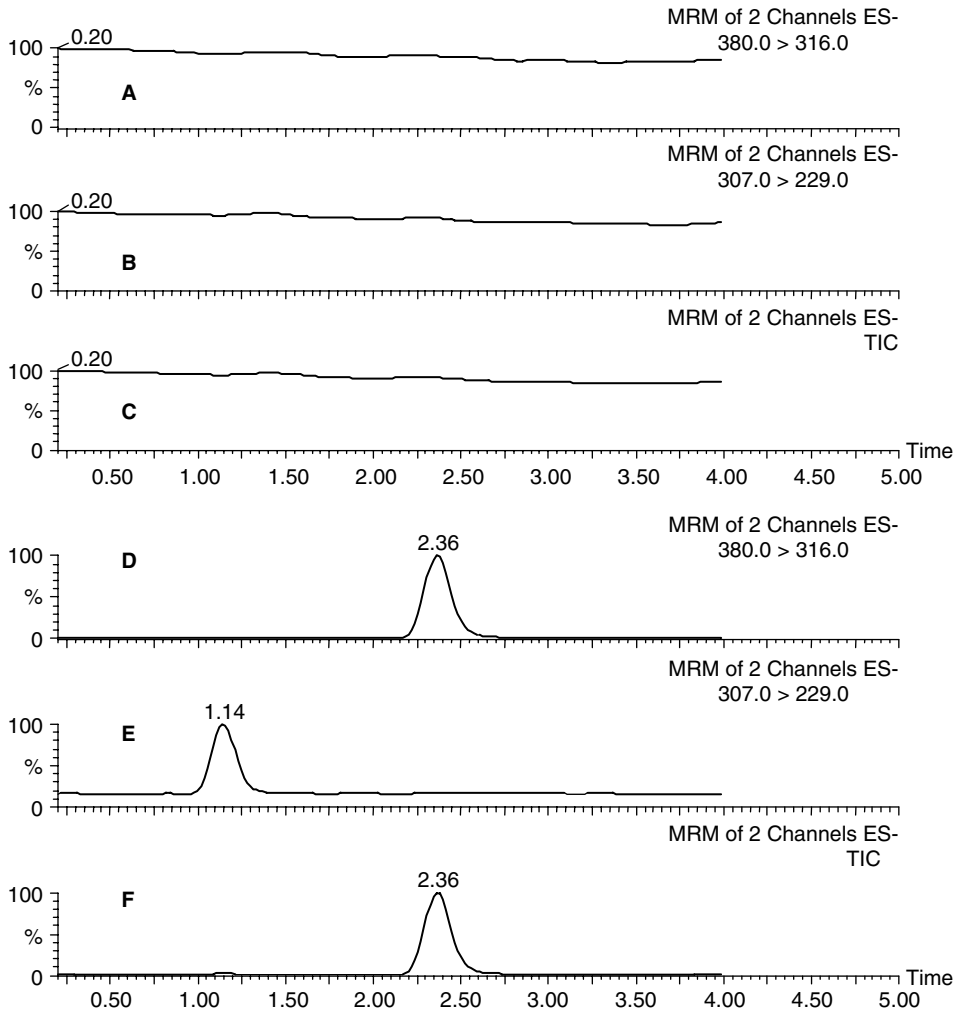


**Figure 4.** Representative ion chromatograms (MRM) illustrating the retention times for analyte and IS (A–C) MRM of two channels ES–: normal blank human plasma. (D–F) MRM of two channels ES–: extracted human plasma spiked with nimesulide (10 ng $ml^{-1}$) and IS (2.0 μg $ml^{-1}$).

room temperature and the plasma was decanted and stored at −20 °C until assayed for their nimesulide content. All samples from a single volunteer were analyzed in the same run in order to avoid inter-assay variations.

## RESULTS AND DISCUSSION

### Assay performance

Linearity, precision and accuracy were determined to assess the performance of method. A linear least-squares regression with a weighting index of $1/x$ was performed on the peak area ratios of nimesulide and IS vs. nimesulide concentrations of the eight human plasma standards (in duplicate) to generate a calibration curve.

A quality control sample (QCA, QCB or QCC) was analyzed after a sequence of 10 unknown samples. The calibration curves showed good linearity within the range 10.0–2000 ng $ml^{-1}$ with a mean correlation coefficient of 0.99492 (Table 1, Fig. 5).

The recovery of nimesulide, based on peak area ratios of extracted normal human plasma/mobile phase, both

**Table 1.** Human plasma calibration curve quality report

| | Concentration (ng $ml^{-1}$) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 10 | 20 | 50 | 100 | 200 | 500 | 1000 | 2000 | $r^2$ |
| Day 1 | 11.0 | 18.1 | 49.0 | 102 | 218 | 470 | 1050 | 1994 | 0.998770 |
| Day 2 | 9.5 | 19.7 | 52.5 | 104.4 | 186 | 502 | 1083 | 1986 | 0.999165 |
| Day 3 | 9.8 | 19.9 | 50.9 | 97.3 | 211 | 490 | 1067 | 1963 | 0.986159 |
| Mean | 10.1 | 19.2 | 50.8 | 101 | 205 | 487 | 1067 | 1981 | 0.99492 |
| SD | 0.8 | 1.0 | 1.8 | 4 | 17 | 16 | 17 | 16 | |
| RSD (%) | 7.7 | 5.1 | 3.5 | 3.6 | 8.2 | 3.3 | 1.6 | 0.8 | |
| RE (%) | 10.1 | 4.8 | 2.0 | 1.0 | 0.5 | 0.2 | 0.1 | 0.0 | |

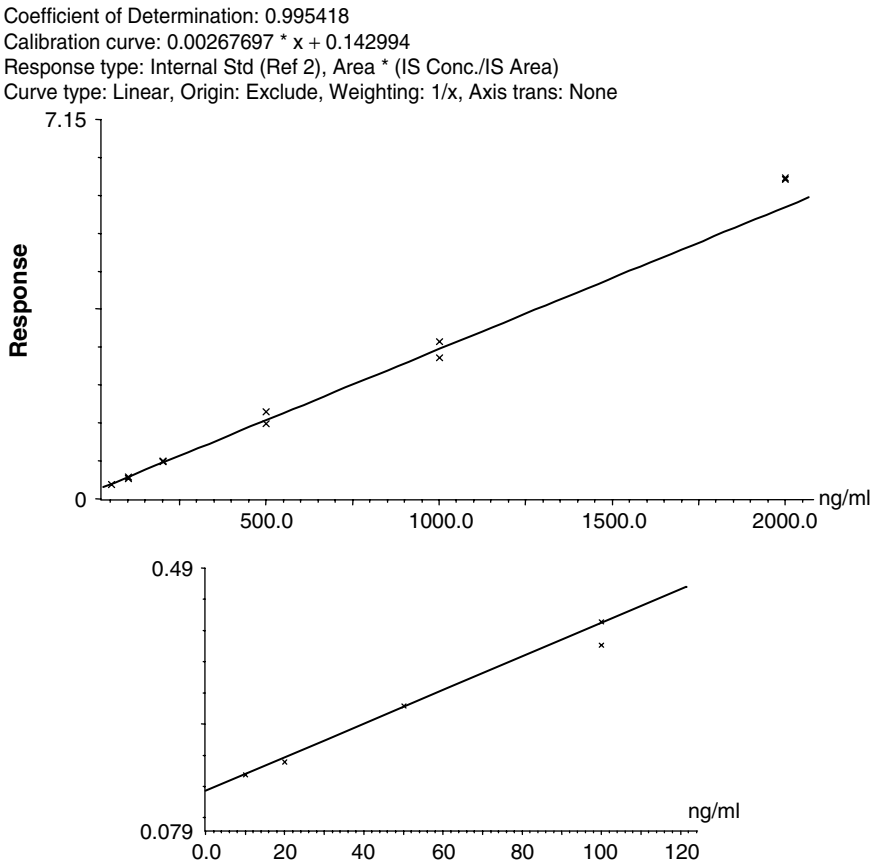


**Figure 5.** Top: calibration curve in the range 10.0–2000 ng ml$^{-1}$. Bottom: expansion of the lower end.

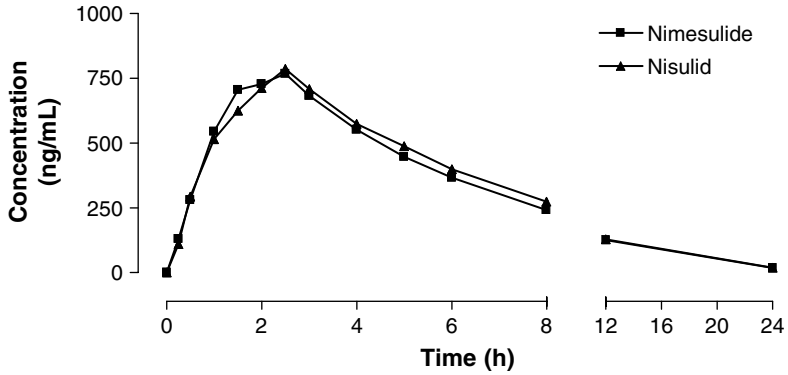


**Figure 6.** Mean plasma nimesulide concentration vs time curves obtained from 24 healthy volunteers following the oral administration of a single 100 mg per 2 ml drop.

previously spiked at a final concentration of 1200 ng ml$^{-1}$, was 97.8 ± 2.3% (mean ± SD, $n = 5$). For the IS (2000 ng ml$^{-1}$) the recovery was 69.8 ± 2.0% (mean ± SD, $n = 10$).

As can be seen in Fig. 2(B) and (C), there is considerably more conversion of parent to product for the analyte than for the IS. Although deuterium-labeled nimesulide would be the ideal IS, celecoxib is commercially available and has similar functional groups to nimusulide.

The assay precision was determined as the relative standard deviation, RSD (%) = 100 (SD/$M$) and the accuracy as the percentage relative error, RE (%) = 100($E - T$)(100/$T$), where $M$ is the mean, SD is the standard deviation of $M$, $E$ is the experimentally determined concentration and $T$ is the theoretical concentration.

The lowest limit of quantification (LOQ) was 10 ng ml$^{-1}$ (see Table 2), defined as the lowest concentration at which both the precision and accuracy were <20%.

The intra- and inter-bach accuracy and precision are shown in Table 2. The intra-bach precision was 6.3–2.1% and the accuracy from 9.9 to 0.1% over the range. The inter-bach accuracy was 3.3, 0.3 and 0.1% for 30, 300 and 1200 ng ml$^{-1}$, respectively, and the precision was 2.3, 2.8 and 2.7%, respectively.

## Stability

Quality control samples (30 and 1200 ng ml$^{-1}$) were subjected to short-term (6 h) room temperature, three freeze–thaw cycles, 24 h autosampler stability and long-term

**Table 2.** Accuracy and precision data for nimesulide from the pre-study validation in plasma

| | Sample | | | | |
|---|---|---|---|---|---|
| Parameter | LOQ | QL2 | QCA | QCB | QCC |
| *Extra-batch* | | | | | |
| Nominal concentration (ng ml$^{-1}$) | 10.0 | 20.0 | 30.0 | 300 | 1200 |
| Mean (ng ml$^{-1}$) | 9.9 | 19.2 | 28.7 | 285 | 1075 |
| SD (ng ml$^{-1}$) ($n = 8$) | 0.5 | 0.6 | 1.5 | 14 | 23 |
| Accuracy (%) | 9.9 | 4.8 | 3.2 | 0.3 | 0.1 |
| Precision (%) | 3.5 | 2.7 | 6.3 | 2.1 | 2.1 |
| *Inter-batch* | | | | | |
| Mean (ng ml$^{-1}$) | 10.5 | 19.2 | 29.4 | 294 | 1108 |
| SD (ng ml$^{-1}$) ($n = 3$) | 0.9 | 0.4 | 0.7 | 8 | 29 |
| Accuracy (%) | 10.5 | 4.8 | 3.3 | 0.3 | 0.1 |
| Precision (%) | 9.2 | 2.0 | 2.3 | 2.8 | 2.7 |

(15 days) stability tests. Subsequently the nimesulide concentrations were measured in comparison with freshly prepared samples and the significance of the results obtained was analyzed using Student's *t*-test ($p > 0.05$).

## Bioequivalence study

The tolerance to both nimesulide formulations was in general good. One volunteer (Vol. XX) complained of headache, but its relation to the drug was not established. Pharmacokinetic parameters are given in Table 3 and mean plasma concentrations of nimesulide are shown in Fig. 6. The maximum concentration reached ($C_{max}$), the area under the curve from the time of dosing to the last measurable concentration ($AUC_{last}$), the area under the curve from the time of dosing to the last common measurable concentration ($AUC_{last\,paired}$) and the area under the curve from the time of dosing extrapolated to infinity ($AUC_{inf}$) were compared.

The nimesulide 50 mg ml$^{-1}$ drop formulation $C_{max}$ geometric mean ratio was 101.0% (90% CI = 93.1–109.6%) of that of the Nisulid 50 mg ml$^{-1}$ drop formulation. The nimesulide 50 mg ml$^{-1}$ drop formulation $AUC_{last}$ geometric mean ratio was 93.6% (90% CI = 87.7–99.8%) of that of the Nisulid 50 mg ml drop formulation. The nimesulide 50 mg ml$^{-1}$ drop formulation $AUC_{last\,paired}$ geometric mean ratio was 95.3% (90% CI = 89.4–101.7%) of that of the Nisulid 50 mg ml$^{-1}$ drop formulation. The nimesulide 50 mg ml$^{-1}$ drop formulation $AUC_{inf}$ geometric mean ratio was 93.7% (90% CI = 88.1–99.7%) of that of the Nisulid 50 mg ml$^{-1}$ drop formulation.

## CONCLUSION

An LC/MS/MS method for the quantification of nimesulide in human plasma was developed and validated. It is in agreement with the high sensitivity, specificity and high sample throughput required for pharmacokinetic studies. Previously reported methods[3,4] have disadvantages such as longer run times (12.0 min) and lower sensitivity (30 ng ml$^{-1}$).

Since the 90% CI for the $C_{max}$ ratio was inside the 80–125% interval proposed by the US Food and Drug Administration,[8,9] it is concluded that the nimesulide 50 mg ml$^{-1}$ drop is bioequivalent to the Nisulid 50 mg ml$^{-1}$ drop with respect to the rate of absorption.

**Table 3.** Pharmacokinetic parameters obtained from 24 healthy volunteers following oral administration of a single 100 mg per 2 ml drop

| | Nimesulide | | | |
|---|---|---|---|---|
| | Nimesulide | | Nisulid | |
| Parameter | Mean | SD (range) | Mean | SD (range) |
| $AUC_{last}$ (ng h ml$^{-1}$) | 5264 | 2784 | 5485 | 2850 |
| $AUC_{last\,paired}$ (ng h ml$^{-1}$) | 5264 | 2784 | 5395 | 2863 |
| $AUC_{Inf}$ (ng h ml$^{-1}$) | 5513 | 2926 | 5718 | 2945 |
| $AUC_{last}$ (0–12 h) /$AUC_{inf}$ (%) | 95.5 | | 95.9 | |
| $AUC_{all}$ (0–24 h) (ng h ml$^{-1}$) | 5423 | 2730 | 5621 | 2774 |
| $AUC_{all}$ (0–24 h) /$AUC_{Inf}$ (%) | 98.4 | | 98.3 | |
| $C_{max}$ (ng ml$^{-1}$) | 866.43 | 284.20 | 851.09 | 265.36 |
| $T_{max}$ (h) | 2.5 | (1.0–4.0) | 2.5 | (0.5–4.0) |
| $T_{1/2}$ (h) | 3.5 | (1.3–8.0) | 3.6 | (1.7–7.0) |
| $K_e$ (h$^{-1}$) | 0.24 | (0.09–0.53) | 0.22 | (0.10–0.41) |

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