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Rapid and simple high-performance liquid chromatographic determination of nimesulide in human plasma

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Abstract

A high-performance liquid chromatographic method for the quantitation of nimesulide in human plasma is presented. The method is based on protein precipitation with methanol and reversed-phase chromatography with spectrophotometric detection at 404 nm. The separation was performed on a Nucleosil 120-5 C_{18}, 50×4-mm I.D. column and the mobile phase consisted of acetonitrile–methanol–15 m*M* potassium dihydrogenphosphate buffer, pH 7.3 (30:5:65, v/v). Only 250 μl of plasma are used for sample preparation and no internal standard is necessary. The limit of quantitation is 80 ng/ml and the calibration curve is linear up to 10 000 ng/ml. More than 20 samples can be analysed within 1 h. Within-day and between-day precision expressed by relative standard deviation is less than 5% and inaccuracy does not exceed 8%. The assay was used for pharmacokinetic studies.

Keywords: Nimesulide

1. Introduction

Nimesulide (Fig. 1) is a nonsteroidal anti-inflammatory drug (NSAID) with antiinflammatory, antipyretic and analgesic properties. Nimesulide seems to cause less severe gastrointestinal side effect compared with other NSAIDs and aspirin [1].

Several HPLC methods have been reported for determination of nimesulide in biological fluids [2–6]. All published methods employ liquid–liquid extraction, separation on reversed-phase columns using acidic mobile phases and detection at 230–290 nm. The limit of quantitation (LOQ) of published procedures varies from 100 to 500 ng/ml but the lower value seems to be necessary for the phar-

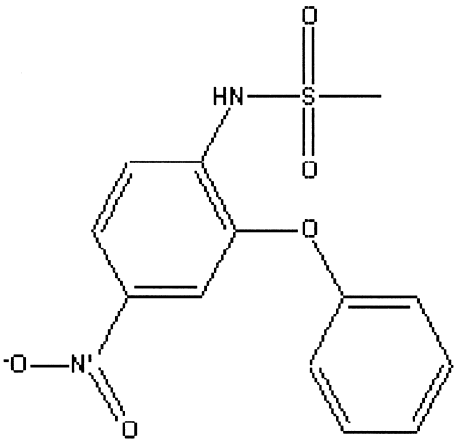

Fig. 1. Structure of nimesulide.

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macokinetic studies with respect to the usually administered dose and corresponding plasma levels.

We present a substantially simplified method which exploits absorbance of light in visible region of the spectrum by nimesulide in solutions at pH 7 and higher. The plasma deproteinisation and detection at 404 nm produces clean chromatograms without interfering and/or lately eluting peaks which results in a short run time of 2 min using a short 5-cm column.

2. Experimental

2.1. Chemicals

Nimesulide was obtained from Léčiva (Czech Republic). Methanol (for chromatography) and potassium dihydrogenphosphate (analytical grade) were manufactured by Merck (Darmstadt, Germany). Acetonitrile (for liquid chromatography) was a Riedel de Haën (Seelze, Germany) product.

2.2. Apparatus

All HPLC instruments were obtained from Thermo Separation Products (Riviera Beach, FL, USA). The system consisted of a membrane degasser, a pump ConstaMetric 4100, an automatic sample injector AS 3000, a spectrophotometric detector UV2000 and a datastation with PC1000 software, version 2.5. The separation was performed on a Nucleosil 120-5 C_{18}, 50×4-mm I.D. column (Watrex, Prague, Czech Republic).

The mobile phase consisted of acetonitrile–methanol–15 m*M* potassium dihydrogenphosphate buffer, pH of the buffer was adjusted to 7.3 with potassium hydroxide (30:5:65, v/v). The flow-rate was 1 ml/min at 30°C. The detection was performed at 404 nm and the time constant was set to 1 s.

2.3. Standards

Stock solutions of nimesulide were made by dissolving of approximately 20 mg in 25 ml of methanol. Separate solutions were prepared for the calibration curve samples and quality control ones. Further solutions were obtained by serial dilutions of stock solutions with methanol. These solutions were added to drug-free plasma in volumes not exceeding 2% of the plasma volume.

All solutions were stored at −18°C and protected from light.

2.4. Preparation of the sample

Two hundred and fifty μl of plasma were pipetted to the tube and 1 ml of methanol was added. The tube was shaken for 30 s at 2000 rpm and centrifuged 5 min at 3500 rpm. A 500-μl aliquot of the supernatant was transferred to the autosampler vial and 500 μl of the buffer (15 m*M* KH_2PO_4, pH adjusted to 7.3 using 1 *M* KOH) were added. The vial was briefly shaken and 50 μl were injected into the chromatographic system.

2.5. Calibration curves

The calibration curve was constructed in the range 80–10 000 ng/ml to encompass the expected concentrations in measured samples. The calibration curves were obtained by weighted linear regression (weighing factor $1/y^2$): the nimesulide peak height was plotted versus nimesulide concentration in ng/ml. The suitability of the calibration model was confirmed by back-calculating the concentrations of the calibration standards.

2.6. Limit of quantitation

Limit of quantitation was defined as the lowest concentration at which the precision expressed by relative standard deviation is better than 20% and accuracy expressed by relative difference of the measured and true value is also lower than 20%. Six identical samples were analyzed for the determination of LOQ.

3. Results and discussion

3.1. Chromatographic conditions and sample preparation

So far published analytical methods for nimesulide determination in plasma use separation on reversed-

phases using acidic mobile phases (pH 3–5.5) and spectrophotometric detection at 230–290 nm. The fact that the nimesulide UV–Vis spectrum is pH dependent has not been analytically exploited yet.

UV–Visible spectrum of nimesulide (Fig. 2) under described conditions fall down monotonously in the 210–300-nm range with only a small shoulder at approximately 270 nm, the intensity of which is about 25% of that at 210 nm. A strong maximum appears at 404 nm at pH>7 and this phenomenon may be used for very selective detection of nimesulide.

The limit of detection was 0.2 ng injected on column in our chromatographic system. This enabled a simple sample preparation based only on protein precipitation without need of internal standard addition.

The retention time of nimesulide was 1.8 min, while the main metabolite, 4-OH-nimesulide was eluted in 0.9 min. The column efficiency expressed by the number of theoretical plates was better than 2000 and peak asymmetry measured at 5% of the peak height was better than 1.3 for nimesulide.

The method selectivity was demonstrated on six blank plasma samples obtained from healthy volunteers: the chromatograms were found to be free of interfering peaks. A typical chromatogram of a blank plasma is shown in Fig. 3. The chromatogram of a plasma sample collected 12 h following administration of 100 mg of nimesulide to a healthy subject is shown in Fig. 4. The measured concentration was 531.4 ng/ml.

3.2. Linearity and limit of quantitation

The calibration curves were linear in the studied range. The mean equation (curve coefficients ±standard deviation) of the calibration curve ($n=6$) obtained from six points was $y=2.66(\pm0.12)x-3.13(\pm8.95)$ with correlation coefficient $r=0.9995$,

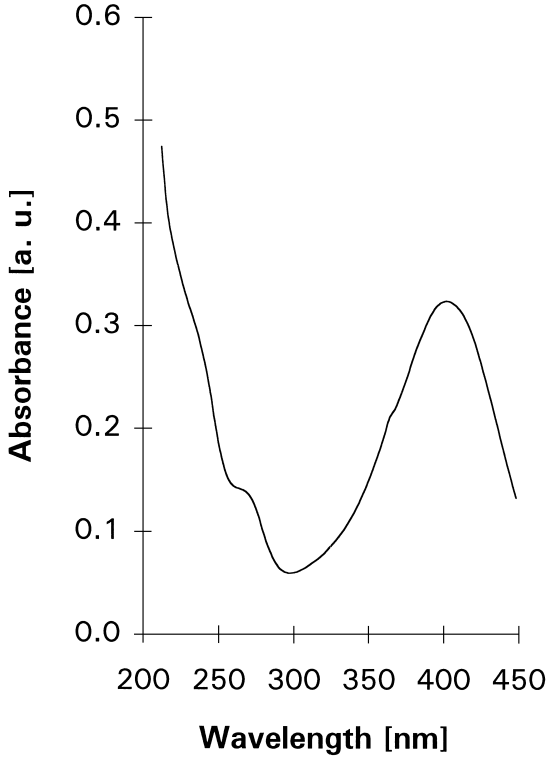

Fig. 2. UV-visible spectrum of nimesulide at pH 7.3.

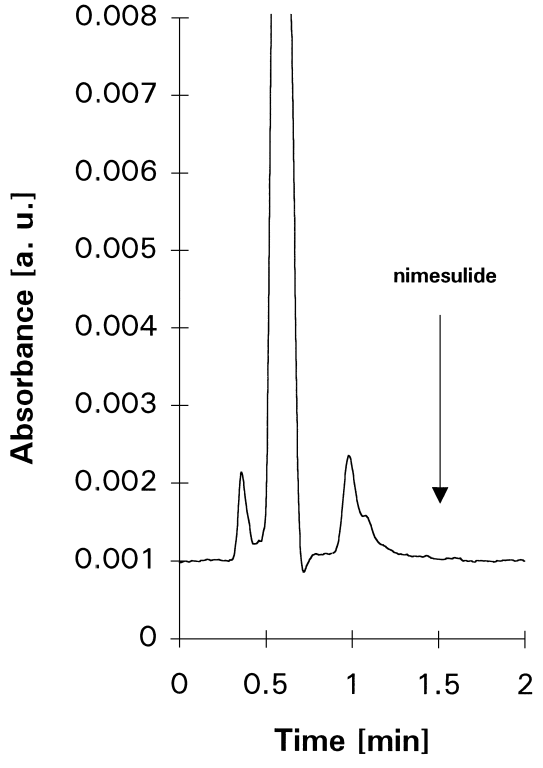

Fig. 3. Typical chromatogram of drug-free human plasma. The arrow indicates the retention time of nimesulide.

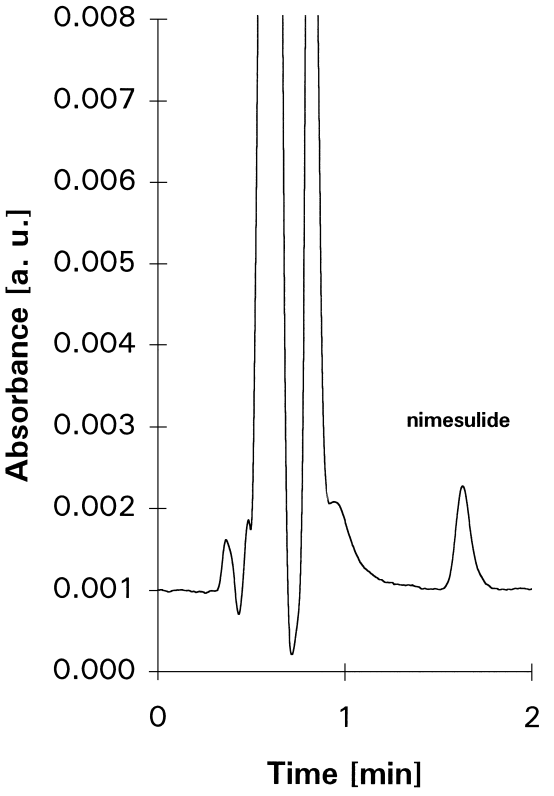

Fig. 4. Chromatogram of a plasma sample from a volunteer 12 h after administration of 100 mg of nimesulide. The respective concentration was 531.4 ng/ml.

where y represents the nimesulide peak height and x represents the nimesulide concentration in ng/ml.

The limit of quantitation was 79.55 ng/ml. The precision, characterised by the relative standard deviation, was 10.0% and accuracy, defined as the deviation between the true and the measured value expressed in percents, was 1.3% at this concentration ($n=6$).

3.2.1. Intra-assay precision

Intra-assay precision of the method is illustrated in Table 1. Six sets of quality control samples (low, medium and high concentration) were analysed with calibration samples in one batch. The precision was better than 5% and accuracy did not exceed 7% at all levels.

Table 1
Intra-assay precision and accuracy

n[a]	Concentration (ng/ml)		Bias (%)	RSD (%)
	Added	Measured		
6	153.0	162.9	6.1	4.5
6	889.5	942.7	5.6	1.7
6	8418	8979	6.3	3.9

[a] n, number of samples.

3.2.2. Inter-assay precision and accuracy

Inter-assay precision and accuracy was evaluated by processing a set of calibration and quality control samples (three levels analysed twice, results averaged for statistical evaluation) in six separate batches. The samples were prepared in advance and stored at −18°C. The respective data are given in Table 2. The precision was better than 4% and the inaccuracy did not exceed 8% at all levels.

3.2.3. Stability study

3.2.3.1. Freeze and thaw stability. Stock solutions of a low and high concentration sample were prepared. The solutions were stored at −18°C and subjected for three thaw–freeze cycles. During each cycle triplicate 0.25-ml aliquots were processed, analysed and the results averaged. The results are shown in Table 3. The concentrations found are within ±6% of nominal concentration, indicating no significant substance loss during repeated thawing and freezing.

3.2.3.2. Processed sample stability. Two sets of samples (153 ng/ml as a low concentration and 8418 ng/ml as a high one of nimesulide) were analysed on 1 day and left in the autosampler at ambient temperature (ca. 25°C). The samples were analysed using a freshly prepared calibration samples 3 days later.

Table 2
Inter-assay precision and accuracy

n[a]	Concentration (ng/ml)		Bias (%)	RSD (%)
	Added	Measured		
6	153.0	164.4	7.5	3.5
6	889.5	893.2	0.4	2.7
6	8418	8396	−0.3	2.5

[a] n, number of days.

Table 3
Stability of the samples

Sample		Cycle 1		Cycle 2		Cycle 3	
C (ng/ml)	*n*[a]	Measured	Bias (%)	Measured	Bias (%)	Measured	Bias (%)
Freeze and thaw stability							
236.5	3	249.7	5.6	249.7	5.6	244.2	3.2
8418	3	8282	−1.6	8585	2.0	8575	1.9
Processed sample stability							
Sample		*C* (ng/ml)	*n*	Conc. found (ng/ml)		RSD (%)	Bias (%)
New		153.0	6	162.9		4.5	6.5
3 Days old		153.0	6	154.5		5.9	1.0
New		8418	6	8979		3.9	6.7
3 Days old		8418	6	8497		4.0	1.0
Long-term stability							
		236.5	6	233.1		3.7	−1.4
		8418	6	8701		1.9	3.4

[a] *n*, number of samples.

The results are presented in Table 3. The processed samples are stable at room temperature for at least 3 days.

3.2.3.3. Long-term stability. Two sets of samples (low and high concentration of nimesulide) were stored in the freezer at −18°C for 6 months. The samples were then analysed using freshly prepared calibration samples. The results are presented in Table 3. The samples are stable at −18°C for at least 6 months.

3.3. Application to biological samples

The proposed method was applied to the determination of nimesulide in plasma samples from the bioequivalence study. Plasma samples were periodically collected up to 24 h after oral administration of 100 mg dose to 26 healthy male volunteers. Fig. 5 shows the mean plasma concentration of nimesulide. The plasma level of nimesulide reached a maximum 1.8 h after the administration and thereafter the plasma level declined with an elimination half-time of ca. 3 h. These values agree with previously published reports [1]. The AUC measured from 0 to the last sampling point was 97% of the value of AUC extrapolated from 0 to infinity which indicates a suitability of the analytical method for pharmacokinetic studies.

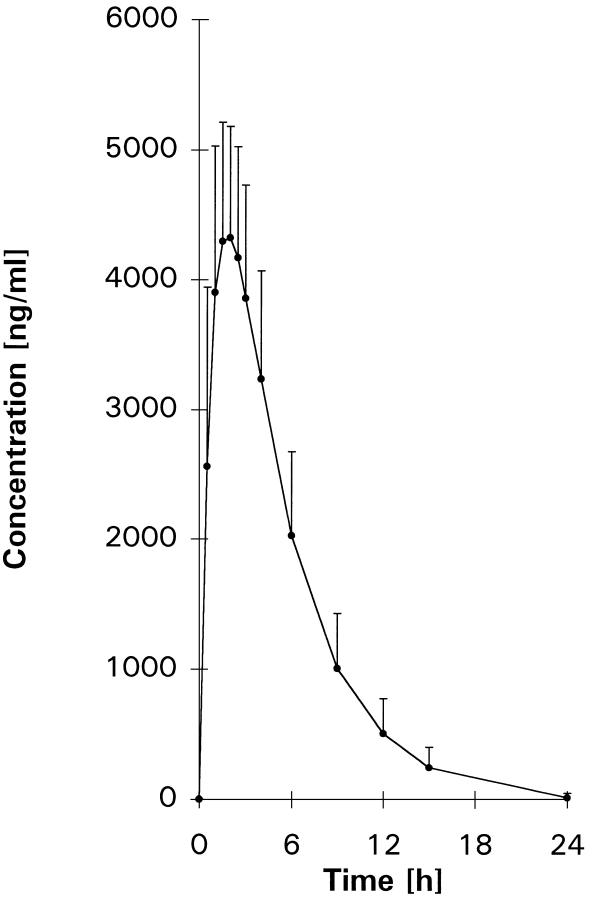

Fig. 5. Mean plasma concentrations (+SD) of nimesulide after 100 mg single oral dose (26 healthy volunteers).

4. Conclusions

The validated method allows determination of nimesulide in the 80–10 000 ng/ml range. Due to the combination of a fast and simple sample preparation with an extremely short analysis time about 300 samples can be measured within 1 day depending on the autosampler capacity. The method is suitable for the pharmacokinetic studies due to its sensitivity and speed.

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