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Determination of Nimesulide and Hydroxynimesulide in Human Plasma by High Performance Liquid Chromatography

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Two specific methods for the simultaneous determination of nimesulide, a non steroidal anti-inflammatory drug, and its hydroxylated metabolite in human plasma are described. Adopting a high performance liquid chromatographic (HPLC) system with UV detection (230 nm), the compounds, extracted from plasma in acidic medium, were separated on ODS columns under gradient conditions, using a phosphate buffer solution and methanol as mobile phase. For each method column length, gradient rate and composition were appropriately selected. The limit of quantitation was 25 ng/mL for both compounds. The two methods were validated by intra-day assays at three concentration levels and applied in kinetic studies in healthy volunteers, during which inter-day assays were carried out confirming their feasibility. © 1998 John Wiley & Sons, Ltd.

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INTRODUCTION

Nimesulide, [4-nitro-2-(phenoxy) methansulphonanilide], is a non steroidal anti-inflammatory drug exerting an antiflogistic, analgesic and antipyretic activity. Showing a good tolerability with a lower incidence of gastrointestinal problems than with other members in its therapeutic class, nimesulide is currently administered in the treatment of several different pathologies (Ward and Brogden, 1988). Its application is found in the treatment of chronic rheumatoid arthritis or osteoarthritis (Fossaluzza and Montagnani, 1989; Magaro *et al*., 1989), inflammation of genitourinary system (Corrado *et al*., 1989; Chiantera *et al*., 1990; Di Leo *et al*., 1990), otorhinolaryngological diseases (Passali *et al*., 1989), odontostomatological practice (Moniaci *et al*., 1988; Solimei *et al*., 1989) and postoperative pain states (Scharli *et al*., 1990; Stefanoni *et al*., 1990). It is usually given in oral doses of 100–200 mg/daily. After single oral administration of the drug to fasting healthy volunteers at the doses of 100 and 200 mg, peak plasma concentrations are reached between 2–4 hours and 1–6 hours, respectively, and account for about 4–9 μg/mL and 6–14 μg/mL. The reported plasma elimination half-life is about 5 hours for both doses (Alessandrini *et al*., 1986; Ward and Brogden, 1988; Gandini *et al*., 1991). The major metabolite hydroxynimesulide, [4-nitro-2-(4′-hydroxyphenoxy) methansulphonanilide] shows a peak level, on average, after 3–8 hours from treatments, with plasma concentrations in the range of about 0.8–2.3 μg/mL (100 mg) and 2.0–4.4 μg/mL (200 mg), and an elimination half-life value of about 5 hours. Following repeated oral doses at 100 mg no significant differences are observed between the parmacokinetic parameters of nimesulide on the 1st and the 7th day (Gandini *et al*., 1991). After oral administration about 70% of the dose is excreted in faeces and the remaining part in urine as hydroxynimesulide metabolite.

Nimesulide detection has been preferably carried out by high-performance liquid chromatography both in plasma (Chang *et al*., 1977) and in pharmaceutical preparations (Nonzioli *et al*., 1989), using common reverse-phase ODS columns. Only in one method has the simultaneous determination of nimesulide and its main metabolite, hydroxynimesulide, in plasma of treated subjects been described (Castoldi *et al*., 1988). By referring to the suggestions proposed in this work, a first improved method was developed, validated and used in a kinetic (bioequivalence) study. Even if this method was not applied in other studies, main problems of interferences from the matrix having emerged in the course of the analyses, its relevant aspects were taken into consideration in developing a second method using new original chromatographic conditions. The latter, demonstrated to be highly feasible, was therefore adopted for other kinetic studies in man. The comparison of the two methods and the statistical results obtained are reported.

EXPERIMENTAL

Chemicals. Nimesulide (code No. N 1016) and tolbutamide (code No. T 0891) were supplied by Sigma-Aldrich, Milan, I. Hydroxynimesulide was synthetically prepared. Confirmatory evidence of its identity was on the basis of IR and NMR spectra. Reagent and solvents, all of analytical or LiChrosolv® grade, were purchased from E. Merck, Darmstadt, D.

0.05 M phosphate buffer, pH 5, used as mobile phase component in Method 1, was prepared by weighing 6.9 g of $NaH_2PO_4 \cdot H_2O$ into a 1 L volumetric flask and diluting to volume with water. The solution was then adjusted to pH 5 with 1N NaOH. 0.05 M phosphate buffer, pH 5.5, used as mobile phase component in Method 2, was prepared by weighing 6.8 g of KH_2PO_4 into a 1 L volumetric flask and diluting to volume with water. The solution was then adjusted to pH 5.5 with 0.05 M K_2HPO_4 solution (8.71 g of K_2HPO_4 to 1 L with water).

Standards. The drugs were dissolved in methanol to yield a stock solution of 1 mg/mL each. Independently prepared stock solutions

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were used for preparation of the working solutions for plasma calibration samples and quality control (QC) samples. Working solutions of mixed nimesulide-hydroxynimesulide were prepared in methanol at concentrations ranging from 100 to 0.5 μg/mL by serial dilution of their respective stock solutions. Those for tolbutamide, i.e. 10 or 50 μg/mL, were similarly obtained. All solutions, prepared monthly, were stored refrigerated in the dark when not in use.

Instrumentation and chromatographic conditions—Method 1. Nimesulide, its hydroxylated metabolite and the internal standard tolbutamide were separated on a LiChrospher® 100 RP-18, 5 μm, 250 × 4 mm i.d. column connected to a LiChrocart® 25–4 manufix containing a LiChrospher® 100 RP-18, 5 μm, guard column (E. Merck, Darmstadt, D), operating at room temperature. Elution was performed under gradient conditions. The original mobile phase, consisting of 0.05 M phosphate buffer, pH 5:methanol 55:45 (v/v), after a run for 3 min was changed to 40:60 (v/v) within 15 min, kept for 8 min, then brought again to the original ratio within 4 min. In a further 2 min the flow was equilibrated. The flow-rate applied by HP 1090 DR5 binary pumps was 1 mL/min and the eluate monitored at 230 nm, by HP 1090 diode array detector (Hewlett–Packard, Milan, I). Injection of the samples (25 μL) was by autosampler built in the instrument. Under these conditions the drugs eluted at about 13.5 min (hydroxynimesulide), 15 min (tolbutamide, I.S.) and 21 min (nimesulide).

Method 2. Separation of the drugs and internal standard was achieved with a Supelcosil LC-18 DB, 3 μm, 33 × 4.6 mm i.d. (Supelchem, Milan, I) connected to a LiChrocart® 25–4 manufix containing a LiChrospher® 100 RP-18, 5 μm, guard column (E. Merck, Darmstadt, D), maintained at 40°C. Elution was performed under gradient conditions. The original mobile phase, consisting of 0.05 M phosphate buffer, pH 5.5:methanol 80:20 (v/v), was changed to 20:80 (v/v) within 16 min, kept for 4 min, then brought again to the original ratio within 5 min. In a further 9 min the flow was equilibrated. The elution solvent was delivered at a flow rate of 0.4 mL/min, monitoring the eluates at 230 nm by diode array detector. Injection of the samples (30 μL) was by autosampler. The method was performed on the same HP 1090 instrumentation. Under these conditions the drugs eluted at about 11.0 min (hydroxynimesulide), 11.7 min (tolbutamide, I.S.) and 13.5 min (nimesulide).

Sample preparation. The preparation of the samples was carried out according to Castoldi *et al*. (1988) with minor modifications, as follows. Plasma samples (1 mL) were adjusted to pH 1 by addition of 30 μL of 12 N hydrochloric acid and spiked with 0.5 μg of internal standard, tolbutamide, (1 μg for Method 2) and 0.2 mL of methanol. After brief whirl-mixing (15 sec), 8 mL of toluene (5 mL for Method 2) were added and the samples extracted on a rotary shaker for 15 min and then centrifuged at 4000 rpm (about 3500 *g*) for 15 min at +4°C. The organic phases were transferred to other tubes and the samples re-extracted as before with 6 mL (4 mL for Method 2) of toluene. The organic phases, pooled with the preceeding ones, were taken to dryness under a nitrogen stream in a water bath at 40°C. The residues were redissolved in 100 μL (200 μL for Method 2) of the original mobile phase, briefly whirl-mixed and transferred to glass vials for automatic injection into the HPLC system.

Calibration and calculation. Blank plasma from untreated healthy human blood donors (AVIS, Turin, I) were used. Evaluation of the assay was performed by seven- to nine-point calibration curves in the concentration range from 25 to 10,000 ng/mL for both nimesulide and hydroxynimesulide. The slope and intercept of the calibration graphs were calculated through weighted ($1/\text{concentration}^2$) linear regression of each drug to internal standard peak-height ratios vs. drug concentration. Experimental peak-height ratios were interpolated on the relative calibration curve and the concentrations back-calculated.

Method validation. The validation of the analytical methods was performed according the suggestions proposed by Shah *et al*., 1991.

Pre-study evaluation—Extraction recovery. Recovery of nimesulide and hydroxynimesulide from plasma was measured by two procedures. The first involved the peak-height measurement of the compounds in extracted samples and in authentic unextracted standards, prepared in mobile phase, spiked at three concentration levels (50, 250 or 500 and 1000 or 2500 ng/mL). The percentage ratio of their peak-heights (extracted vs. unextracted) was taken as value of extraction recovery. Average values were calculated at each concentration and at all concentrations tested. The second involved the evaluation of the slopes obtained in analysis of their respective regression lines. The percentage ratios of the slopes (extracted vs. unextracted) was assumed as second value of extraction recovery, and the mean values calculated. Overall values of recovery were obtained averaging the results by the two procedures.

Limit of quantitation (LOQ, plasma spiked samples). Replicate analyses ($n = 5$) of plasma samples, spiked at 25 and 50 ng/mL, were performed. The percentage ratio between the mean concentration obtained and the nominal one was assumed as accuracy parameter, while the mean coefficient of variation percentage was assumed as precision value. The results were accepted when the accuracy values were in the range 80–120% and those of precision were $\leqslant 20\%$.

Accuracy and precision (plasma spiked samples). Intra-day assay at concentrations higher than LOQ was performed on freshly prepared plasma samples, spiked at 50, 250 and 1000 (or 2500) ng/mL, ($n = 5$/concentration). Acceptance criteria of the results were based on accuracy values in the range from 85 to 115% and precision values $\leqslant 15\%$.

Stability in the autosampler (plasma spiked samples). Aliquots of the above-mentioned spiked samples remained capped in the autosampler at room temperature for 24 hours before injection in order to verify their stability over the longest expected period of the unknown samples in the autosampler. The results were compared with the preceding ones and evaluated according to the same acceptance limits.

Within-study evaluation—Linearity. Intercept, slope and coefficient of correlation (R) were evaluated for each calibration curve performed daily. The calibration was accepted if the R value found was above the tabulated one corresponding to the significant level $p = 0.01$ for the n calibration points and n-2 degrees of freedom. Mean, S.D. and CV% values of the slope and R parameters were also calculated.

Accuracy and precision (plasma calibration samples). The concentration value of each calibration point was back-calculated from the equation of the corresponding calibration curve, performed daily with the unknown samples. The results were accepted/rejected according to the preceding evaluation criteria (at LOQ: accuracy 80–120% and precision $\leqslant 20\%$; at the other concentrations: accuracy 85–115% and precision $\leqslant 15\%$). The

Table 1. Pre-study validation in nimesulide and hydroxynimesulide assay. Statistical values Recovery percentage ($n = 5$/ concentration; $n = 15$ for I.S.)

	Method 1						Method 2					
Nominal concentration	Nimesulide			Hydroxynimesulide			Nimesulide			Hydroxynimesulide		
(ng/mL)	Mean	S.D.	CV%	Mean	S.D.	CV%	Mean	S.D.	CV%	Mean	S.D.	CV%
50	104.1	20.7	19.9	86.4	10.1	11.7	79.9	10.3	12.8	89.2	9.7	10.9
250	99.9	7.0	7.0	78.1	6.4	8.2						
500							77.2	8.4	10.8	79.4	7.2	9.0
1000	99.3	5.5	5.5	83.8	6.0	7.2						
2500							81.7	11.8	14.4	81.4	11.0	13.5
Mean recovery												
by peak height	101.1	12.2	12.1	82.8	8.0	9.6	79.6	9.7	12.1	83.3	9.8	11.7
by slope (*)	97.9	4.9	5.0	82.6	15.0	12.4	92.8	10.2	9.4	90.7	10.4	9.5
Overall mean recovery	99.5			82.7			86.2			87.0		
			Tolbutamide (I.S.)						Tolbutamide (I.S.)			
500	94.4	7.4	7.8									
1000							87.9	7.5	8.6			

(*) regression equations (average values):

Method 1	Extracted	nimesulide:	$Y = 0.00143X - 0.00849$	$r = 0.99855$
	Authentic	nimesulide:	$Y = 0.00146X - 0.00077$	$r = 0.99748$
	Extracted	hydroxynimesulide	$Y = 0.00157X - 0.01102$	$r = 0.99796$
	Authentic	hydroxynimesulide	$Y = 0.00190X + 0.00213$	$r = 0.99899$
Method 2	Extracted	nimesulide	$Y = 0.00065X + 0.00026$	$r = 0.99887$
	Authentic	nimesulide	$Y = 0.00070X + 0.00109$	$r = 0.99389$
	Extracted	hydroxynimesulide	$Y = 0.00078X + 0.00544$	$r = 0.99621$
	Authentic	hydroxynimesulide	$Y = 0.00086X + 0.00969$	$r = 0.99495$

mean values obtained were statistically evaluated as inter-day assay for plasma calibration samples.

Accuracy and precision (quality control samples). Before starting the analysis of the unknown samples, separate aliquots of blank plasma samples, spiked at three concentration levels, namely 100 (or 50), 500 and 2500 ng/mL of nimesulide and 50, 250 (or 500) and 1000 (or 2500) ng/mL of hydroxynimesulide, were prepared and stored frozen. Two replicates/concentration were thawed daily and analysed with a complete calibration curve along with unknown samples. The analysis of the unknown samples was accepted if (i) at least 4 of the 6 QC samples were found within ±20% of their nominal values and (ii) the 2 possible QCs outside ±20% of their nominal values were not both at the same nominal concentration. The mean, S.D. and CV% values obtained were considered as inter-day assay for plasma QC and the same statistical criteria of evaluation as those in plasma spiked and calibration samples were applied.

RESULTS AND DISCUSSION

Even though nimesulide is not a recent drug, reference analytical works on it are scarce. In the development of our first analytical method an idea was nevertheless taken from the relevant work of Castoldi *et al*., 1988. Some of their suggestions, such as the extraction procedure of the plasma samples, the separation of nimesulide and its hydroxylated metabolite on an ODS column and the wavelength selected, were in fact considered preliminary to further improvements to be made. However, in the practical application, the compounds were seen to overlap endogenous peaks of the matrix when an isocratic elution was used. Therefore a gradient elution was attempted using a column of similar phase (ODS), but the particles of smaller size (5 μm) to increase resolution. This proved to be effective on the separation of hydroxynimesulide from any other interference, but not on that of nimesulide, which, independently of the analytical conditions adopted, always eluted on a minor interfering peak of the matrix both when testing different blank plasma samples from pooled batches or from volunteers before treatment. These interferences consequently reflected (i) on the recovery of nimesulide showing, on average, overestimated percentages (Table 1) and (ii) on its limit of quantitation, that resulted 50 ng/mL (instead of the expected 25 ng/mL, as for hydroxynimesulide; the evaluation of nimesulide at this concentration resulted highly inaccurate, i.e., 144%, (Table 2). The extraction recoveries for its metabolite and internal standard were however in agreement with those reported by Castoldi *et al*., 1988. The extraction procedure involved the use of the less toxic toluene than benzene and setting the water bath temperature at 40°C to take the extracts to dryness. This evaporation condition was suggested by the fact that, in preliminary testing, some samples showed a noticeable and not reproducible loss of drugs and internal standard when processed under slow evaporation at room temperature (as reported in the original method) or quick dried at 60°C. This fact was tentatively explained by the concomitant effects of bath temperature, gas flow-rate and time the sample remains dry that could produce uncontrollable evaporation and/or absorption on the glass walls of the tubes, which are different for each drug tested. This very critical step of the procedure was perfected only by carefully controlling the nitrogen flow-rate and evaporation time, and completing the process by visual observation of the samples. As a final result, chromatograms like those shown in Fig. 1 were obtained. In spite of these difficulties and the limits presented by this first method, the intra-day analyses carried out in the pre-study validation confirmed its reproducibility, the data obtained on accuracy and precision satisfying the

Table 2. Pre-study validation in nimesulide and hydroxynimesulide assay. Statistical values.

Limit of quantitation (LOQ): plasma spiked samples ($n=5$)

	Method1		Method 2
	Nominal concentration (ng/mL)		
	25	50	25
	Nimesulide concentration found (ng/mL)		
Mean	36.0	48.2	25.6
S.D.	4.9	4.2	0.2
CV% (precision)	13.7	8.7	0.7
Accuracy (%)	144.0	96.4	102.2
	Hydroxyimesulide concentration found (ng/mL)		
Mean	23.8		24.2
S.D.	4.3		1.0
CV% (precision)	17.9		4.2
Accuracy (%)	95.2		96.7

Acceptance criteria (Shah *et al.*, 1991): accuracy: 80–120%; precision: ⩽20%

Intra-run accuracy and precision (plasma spiked samples) and stability in the autosampler (24 h) ($n=5$).

	Nominal concentration (ng/mL)									
	50		250		500		1000		2500	
	Nimesulide concentration found (ng/mL)									
	Method 1									
	Fresh	24h	Fresh	24h	Fresh	24h	Fresh	24h	Fresh	24h
Mean	48.2	49.6	260.4	265.0			1005.2	1007.6		
S.D.	4.2	9.5	15.6	12.7			51.3	44.1		
CV% (precision)	8.7	19.2	6.0	4.8			5.1	4.4		
Accuracy (%)	96.4	99.2	104.2	106.0			100.5	100.8		
	Method 2									
Mean	49.8	50.2			528.8	514.6			2653.0	2648.4
S.D.	2.2	3.3			32.8	31.2			210.7	206.3
CV% (precision)	4.2	4.2			6.3	6.1			7.9	7.8
Accuracy (%)	99.6	99.6			104.8	102.9			106.1	105.9
	Hydroxynimesulide concentration found (ng/mL)									
	Method 1									
Mean	50.2	51.4	253.2	254.6			1027.4	1034.2		
S.D.	7.6	5.4	25.3	24.0			112.8	102.7		
CV% (precision)	15.1	10.4	10.0	9.4			11.0	9.9		
Accuracy (%)	100.4	102.8	101.3	101.8			102.7	103.4		
	Method 2									
Mean	50.4	50.2			504.0	489.6			2356.8	2301.2
S.D.	0.5	0.8			18.9	19.0			103.3	85.8
CV% (precision)	1.0	1.8			3.7	3.9			4.4	3.7
Accuracy (%)	100.7	100.7			100.8	97.9			93.1	92.0

Acceptance criteria (Shah *et al.*, 1991): accuracy: 85–115%; precision: ⩽15%

pre-defined acceptance criteria (Table 2). Therefore the method was applied in the analysis of authentic plasma samples taken from volunteers in a kinetic study during which more than 300 samples were analysed and a complete within-study assay performed. A good linearity was found over the entire range of calibration curves (50–5000 ng/mL, nimesulide; 25–2500 ng/mL, hydroxynimesulide; $n=11$), their coefficients of correlation (R) ranging from 0.99461 to 0.99952 (nimesulide) and from 0.98893 to 0.99916 (hydroxynimesulide). All the runs were accepted, the R values being above the significance level $p=0.01$ for 6 calibration points and 4 degrees of freedom (i.e., 0.917 for nimesulide) and 5 calibration points and 3 degrees of freedom (i.e., 0.959 for hydroxynimesulide). The slope values remained quite similar to those obtained in recovery testing, thus indicating that the method was reproducible (Tables 1 and 3). The back-calculated concentrations of the calibration samples resulted, on average, within the acceptance criteria, confirming the limits of quantitation previously observed (50 ng/mL for nimesulide and 25 ng/mL for hydroxynimesulide, Table 3). No runs were rejected, the results obtained in analysis of the quality control samples, injected daily with unknown samples, complying with the assumed acceptance criteria (Table 4). Only 9, for nimesulide, and 7, for hydroxynimesulide, of 66 QC samples analysed were found to be outside them.

Even if the method appeared on the whole highly feasible and the favourable analytical conditions gave rise to very good separation, nevertheless it was not used in other studies. The reason was that in the course of the analyses of unknown plasma samples some subjects presented chromatographic profiles quite different from those observed analysing plasma batches from human donors, i.e., showing unexpected relevant interfering peaks in the area of nimesulide and hydroxynimesulide elution, that required subtraction from their relative blanks. This fact was the main reason for which it was decided to develop a new analytical method for other kinetic studies, taking into account the experience acquired in these analyses.

The new approach was attempted adopting a specific ODS column for basic drugs, containing particles of reduced size (3 μm), flowing at low rates and under gradient

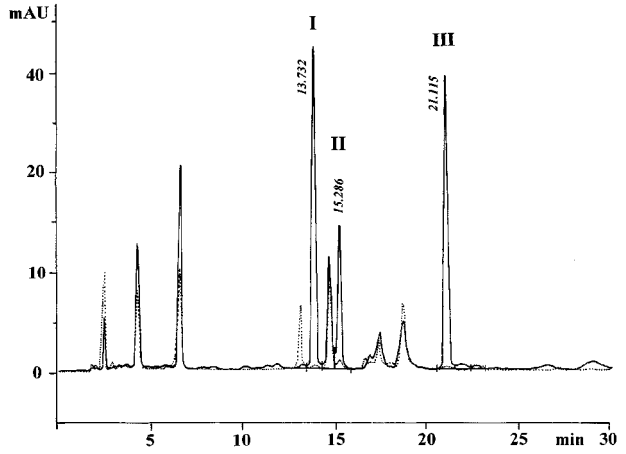

Figure 1. Chromatographic traces obtained in analysis of authentic plasma samples taken from a subject before treatment (dotted line) and 4 hours after a single oral administration of nimesulide, 100 mg sachet (continuous line). Conditions (Method 1): Column: LiChrospher® 100 RP18, 5 μm, 250×4 mm, room temperature; Eluent: 45% B, steady 3 min, to 60% B at 1%/min, steady 8 min, 1 mL/min. [A]: 0.05 M phosphate buffer, pH 5, [B]: methanol; Detector: diode array at 230 nm. Peak identification: (I) Hydroxynimesulide (1322 ng/mL), (II) Tolbutamide, internal standard (500 ng/mL) and (III) Nimesulide (1677 ng/mL).

conditions. The original mobile phase consisted of methanol (from 30 to 80%) and phosphate buffer (0.05 M K_2HPO_4) solution. This was prepared at different pHs, i.e., at pH 3 with H_3PO_4 or at pH 5 with triethylamine or at pH 7 with KH_2PO_4. The best separations were achieved when using solutions buffered at about pH 5, as in the first method. These conditions avoided obtaining distorted, very wide peaks, eluted too early (within 6 min, as at pH 3), or overlapping the matrix peaks (as at pH 7). Adjustment of the pH of the buffer solution was successively done with 0.05 M KH_2PO_4 solution instead of triethylamine, giving more uniform retention times of the test compounds. An improvement in the separation and shape of the peaks was made by setting the flow-rate within 0.3 and 0.5 mL/min, and

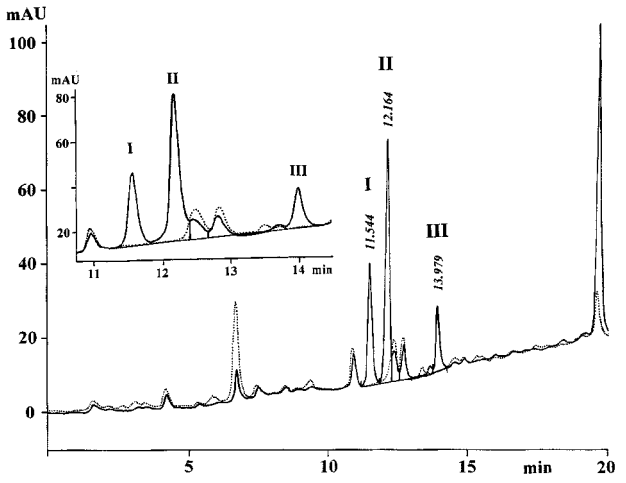

Figure 2. Chromatographic traces obtained in analysis of authentic plasma samples taken from a subject before treatment (dotted line) and 2 hours after a single oral administration of nimesulide, 100 mg tablet (continuous line). Conditions (Method 2): Column: Supelcosil LC-18 DB, 3 μm, 33×4.6 mm, 40°C; Eluent: 20–80% B at 3.75%/min, steady 4 min, 0.4 mL/min. [A]: 0.05 M phosphate buffer, pH 5.5, [B]: methanol; Detector: diode array at 230 nm. Peak identification: (I) Hydroxynimesulide (821 ng/mL), (II) Tolbutamide, internal standard (1000 ng/mL) and (III) Nimesulide (710 ng/mL).

preferably using a longer guard column (4 cm instead of 1 cm) and thermostatted conditions (above 35°C). Furthermore, it was observed that the gradient effect (i.e., when the real composition of the gradient entered the column and was effective on peak separation) occurred 3–5 min later than that indicated by the mixing of the pumps. These considerations permitted the final separation to be planned starting directly with a gradient programme, without an isocratic pre-run, enriching the initial composition of the mobile phase with phosphate buffer solution (80%) to elute the more hydrophilic compounds of the matrix in the first part of the chromatogram (within 6–7 min) until reversing the ratio with the methanol to elute all other compounds having more 'organic' character. The methanol percentage delivered in the gradient programme, was 3.75 %/min, sufficient to avoid a rapid drift of the baseline and to elute the tested samples in reasonable times. The run time took 30 min for a complete analysis, the chromatographic system involving the use of a reduced flow and thus requiring a long time to re-equilibrate the column to the original conditions.

In order to evaluate the possible interferences from the matrix, several plasma batches, as well some of the samples that presented analytical problems with the preceding method, were reassayed. It was confirmed that the plasma batches are a more problematic source of endogenous peaks (also different between batches) than the unknown samples themselves. However, under the analytical conditions adopted, no peaks were presented in the elution areas of nimesulide and hydroxynimesulide, as can be seen from the chromatograms presented in Fig. 2, while the internal standard peak eluted with a shoulder, of minor relevance, that nevertheless did not interfere with the correct measurement of the baseline. To minimize this shoulder peak, the amount of internal standard added was increased to 1 μg.

A recovery test was again performed decreasing the amount of solvent used in extraction to limit the difficulties occuring in the evaporation step. The percentages of the drugs (Table 1) resulted lower than those in the first method, but were considered more realistic and uniform (about 87% for both compounds and internal standard), the measurement of the peaks occurring without interferences. Even if the extraction procedure was less effective, nevertheless the method resulted more sensitive than the preceding one (the extracts were dissolved in 200 μL of the mobile phase instead of 100 μL, the amount injected remaining practically the same). As a consequence, the limit of quantitation for both nimesulide and hydroxynimesulide resulted 25 ng/mL (while in Castoldi *et al.*, 1988, it was 50 ng/mL), a value reached with high accuracy and precision (Table 2). From a general point of view, the intra-day assay test, carried out analysing samples, spiked at three concentration levels, either freshly prepared or stored in the autosampler (Table 2), indicated that this second method was more precise than the first one, while both methods appeared similarly accurate.

The favourable results obtained by the second method in the preliminary assays were reinforced by those obtained applying it to the analysis of the unknown samples taken from other two kinetic studies in man, in which more than 1000 samples, including calibration and quality control samples, were analysed. Since the data collected in the course of the two studies were highly comparable, all the results obtained were taken into consideration in order to give more significance to the statistical evaluation of the within-study validation of this method. The overall considerations are therefore discussed hereafter.

Table 3. Within-study validation in nimesulide and hydroxynimesulide assay. Statistical values.

Plasma calibration curves, descriptive statistics

	Method 1 (n=11)				Method 2 (n=27)			
	Nimesulide		Hydroxy nimesulide		Nimesulide		Hydroxy nimesulide	
	Slope (ng/mL)$^{-1}$	R	Slope (ng/mL)$^{-1}$	R	Slope (ng/mL)$^{-}$	R	Slope (ng/mL)$^{-1}$	R
Mean	0.00131	0.99700	0.00167	0.99673	0.00045	0.99737	0.00068	0.99792
S.D.	0.00016	0.00150	0.00023	0.00284	0.00006	0.00125	0.00008	0.00093
CV%	12.0		13.8		12.8		11.8	

Inter-run accuracy and precision (plasma calibration samples).

	Nominal plasma concentration (ng/mL)								
	25	50	100	250	500	1000	2500	5000	10000
	Back-calculated Nimesulide plasma concentration (ng/mL)								
	Method 1								
Mean		50.9	95.1	258.5	511.2	954.6	2509.5	5050.6	
S.D.		2.1	7.4	12.8	25.7	52.5	90.9	358.5	
CV% (precision)		4.2	7.9	4.9	5.0	5.5	3.6	7.1	
Accuracy (%)		101.6	95.1	103.5	102.2	95.4	100.4	101.0	
n		10	10	11	11	10	11	11	
	Method 2								
Mean	25.0	49.9	99.6	253.7	506.7	999.2	2535.3	4913.4	9399.6
S.D.	0.6	2.8	7.1	15.3	31.2	53.3	156.7	318.7	575.8
CV% (precision)	2.4	5.6	7.1	6.0	6.2	5.3	6.2	6.5	6.1
Accuracy (%)	100.0	99.8	99.6	101.5	101.3	99.9	101.4	98.3	94.0
n	25	25	26	25	27	26	27	27	8
	Back-calculated Hydroxynimesulide plasma concentration (ng/mL)								
	Method 1								
Mean	25.0	50.0	100.2	256.9	513.7	947.3	2483.1		
S.D.	0.8	4.4	9.9	12.5	19.7	61.6	121.9		
CV% (precision)	2.8	8.3	9.8	4.9	3.9	6.5	4.9		
Accuracy (%)	99.6	100.2	100.3	102.8	102.8	94.7	99.3		
n	10	5	9	11	11	10	11		
	Method 2								
Mean	25.1	50.1	98.3	249.1	506.5	1006.9	2515.6	5032.7	9463.2
S.D.	1.1	3.7	5.1	14.1	19.6	44.8	125.8	246.0	501.7
CV% (precision)	4.3	7.4	5.2	5.6	3.9	4.4	5.0	4.9	5.3
Accuracy (%)	100.3	100.3	98.3	99.6	101.3	100.7	100.6	100.7	94.6
n	27	27	26	25	27	27	26	27	9

Acceptance criteria (Shah *et al.*, 1991): at the lowest concentration, accuracy: 80–120%; precision: ⩽20%: at the other concentrations, accuracy: 85–115%; precision: ⩽15%

siderations are therefore discussed hereafter.

During these analyses good linearity was found over the entire ranges of the calibration curves. The ranges were extended from 25 to 5000 ng/mL of both drugs in 16 of the 27 runs cumulatively carried out and from 25 to 10,000 ng/mL in 11 runs (while those reported by Castoldi *et al.*, 1988, were in the ranges of 250–5000 ng/mL (numesulide) and 100–2500 ng/mL (hydroxynimesulide)). As a result, the coefficients of correlation ranged from 0.99302 to 0.99940 (nimesulide) and from 0.99643 to 0.99939 (hydroxynimesulide). All the runs were thus accepted, the R values all being above the tabulated value of 0.874, corresponding to the significance level $p=0.01$ for the minimum number of samples considered in calibrating both drugs, i.e., 7 calibration points and 5 degrees of freedom. Accuracy and precision data, evaluated on the back-calculated concentrations, fully satisfied the predefined acceptance criteria both at the limit of quantitation (25 ng/mL) and at the other concentrations tested (Table 3). The excellent results obtained in evaluation of the quality control samples confirmed the reproducibility of the method (Table 4). In fact, accuracy levels ranged from 100.6 to 102.7% (nimesulide) and from 100.7 to 103.1% (hydroxynimesulide), while the precision ranges were from 7.7 to 14.4% (nimesulide) and from 6.3 to 10.1% (hydroxynimesulide), i.e., all well within the predefined acceptance criteria. Nimesulide concentrations, in the 162 QC samples cumulatively analysed daily with the unknown samples, were found all within the acceptance intervals (±20% of the nominal concentrations) in 21 of the 27 runs. In 5 runs 1 QC per run (1 at 500 ng/mL and 4 at 2500 ng/mL) and in 1 run 2 QCs (1 at 500 ng/ml and 1 at 2500 ng/mL) were found outside these acceptance criteria. Those for hydroxynimesulide were found all within the acceptance intervals in 26 of the 27 runs (in 1 run only 1 QC at 50 ng/mL was found outside). Therefore all the analytical runs were accepted.

Finally, since the work required analysing unknown samples over a long period of time, a stability test was also performed. Before starting the analysis of unknown samples, blank plasma samples were spiked at 50 and 2500 ng/mL with nimesulide+hydroxynimesulide and stored frozen at −20°C. At the end of the studies, i.e., about 10 months later (295 days), five samples/concentration were thawed and analysed in parallel with freshly spiked plasma samples.

The recoveries found at the two concentrations were

Table 4. Within-study validation in nimesulide and hydroxynimesulide assay. Statistical values. Inter-run accuracy and precision (plasma quality control samples)

	Nominal plasma concentration (ng/mL)					
	50	100	250	500	1000	2500
	Nimesulide concentration found (ng/mL)					
	Method 1					
Mean		102.1		492.3		2415.3
S.D.		13.0		66.0		269.8
CV% (precision)		12.6		13.4		11.2
Accuracy (%)		102.0		98.5		96.6
n		22		19		22
	Method 2					
Mean	50.3			506.6		2566.5
S.D.	3.9			57.0		369.0
CV% (precision)	7.7			11.3		14.4
Accuracy (%)	100.6			101.3		102.7
n	53			53		53
	Hydroxynimesulide concentration found (ng/mL)					
	Method 1					
Mean	51.4		254.2		972.5	
S.D.	5.3		23.4		95.3	
CV% (precision)	10.3		9.2		9.8	
Accuracy (%)	102.5		101.7		97.2	
n	22		19		22	
	Method 2					
Mean	51.5			507.3		2516.8
S.D.	5.2			32.0		221.1
CV% (precision)	10.1			6.3		8.8
Accuracy (%)	103.1			101.5		100.7
n	53			53		53

Acceptance criteria (Shah *et al.*, 1991): accuracy: 85–115%; precision: $\leqslant$15%

respectively 102.0 and 103.0% for nimesulide, and 100.0 and 101.0% for hydroxynimesulide, confirming that no degradation of either drug occurred.

CONCLUSION

The applicability of both methods was evaluated in the analysis of unknown samples taken from volunteers in kinetic studies. Both methods demonstrated to be highly feasible and reproducible. The choice to use preferably one of them on further applications will mainly involve an appropriate evaluation of the matrix interferences. Consequently, this choice could also depend on the need to reach higher sensitivity at LOQ. In this case, the second method developed would be preferable, having been demonstrated to be more sensitive than the first one.

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