ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
18 (1998) 201–211

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Mass spectrometric characterization and HPLC determination of the main urinary metabolites of nimesulide in man[1]

Marina Carini *, Giancarlo Aldini, Rita Stefani, Cristina Marinello, Roberto Maffei Facino

Istituto Chimico Farmaceutico Tossicologico, University of Milan, Viale Abruzzi 42, 20131 Milan, Italy

Received 20 October 1997; received in revised form 16 December 1997; accepted 1 January 1998

Abstract

A study was undertaken for the characterization and quantitative determination of the main urinary metabolites of the non-steroidal anti-inflammatory drug (NSAID) nimesulide (4-nitro-2-phenoxy-methanesulfonanilide) in man following single oral administration (200 mg). Urines were collected from six healthy volunteers at 12, 24, 48, 72 and 96 h post-administration and submitted to liquid–liquid extraction before (free metabolites) and after enzymatic hydrolysis (conjugated metabolites). The structure of the metabolites, isolated by TLC separation, was elucidated by mass spectrometry (electron impact ionization) and confirmed by synthesis. Five metabolites were identified: they arise from hydroxylation to the phenoxy nucleus (M1 = hydroxynimesulide); reduction of the nitro group to an amino derivative (M2); concomitant hydroxylation and reduction (M3); *N*-acetylation of the M2 (M4) and of the M3 (M5) metabolites. Quantitation was by reverse phase high performance liquid chromatography (Supelcosil LC-18 DB column; mobile phase: sodium phosphate buffer (pH 3.0, 50 mM)-acetonitrile (gradient elution); flow rate: 1 ml min^{-1}; UV detection, 230 nm), procedure which allows in a single chromatographic run the simultaneous determination of the unchanged drug and of its metabolites. The urinary excretion of the drug and metabolites (free + conjugated) in the overall 96 h-interval accounts for approximately 40% of the administered dose: $17.55 \pm 3.6\%$ M1; $0.72 \pm 0.43\%$ M2; $2.45 \pm 1.22\%$ M3; $19.07 \pm 4.3\%$ M5. The bulk of the metabolites was in conjugated form. Percentages excretion of the unchanged drug and of M4 metabolite were below 0.5%. The described method is suited to specifically and quantitatively measure nimesulide and metabolites in human urine with acceptable precision and accuracy.

Keywords: Nimesulide; NSAID; Human urines; Metabolites characterization (EI-MS); Reverse-phase liquid chromatography; Urinary levels of metabolites

1. Introduction

Nimesulide, 4-nitro-2-phenoxy-methanesulfonanilide is a well tolerated non-steroidal anti-infl-

* Corresponding author. Fax: +39 2 29510823; e-mail: Marina.Carini@unimi.it

[1] Presented at the 7th Meeting on Recent Developments in Pharmaceutical Analysis, Island of Elba, Italy, September 16–20, 1997.

0731-7085/98/$ - see front matter
PII S0731-7085(98)00172-1

ammatory drug with well demonstrated antipiretic and analgesic properties. Notwithstanding the drug is currently and successfully used in the treatment of a wide variety of painful inflammatory diseases [1], and is marketed in a large number of European and extra European countries, there are no published studies on its metabolism in man.

The only available study reports the formation and quantitation in urine and plasma of only one metabolite, identified as the hydroxylated derivative of the drug to the phenoxy ring: 4-hydroxynimesulide, but it does not give information about the overall biotransformation pathways [2]. In a pilot experiment performed by us on human volunteers, another metabolite, 2-[(4′-hydroxy) phenoxy]-4-*N*-acetylamino-methane-sulfonanilide was identified in human urines, but its characterization (mass spectrometric confirmation) and quantitative determination in urines have never been published. Subsequent studies in vitro using rat liver subcellular fractions confirm the formation of hydroxynimesulide (a reaction catalyzed by the cytochrome P-450 family), but also indicate the presence of other metabolic species [3].

Therefore, the aim of the present work was to study the biotransformation pathways of nimesulide in man after acute administration of the drug, following isolation and mass spectrometric characterization of its main urinary metabolites. In addition, a sensitive and specific HPLC assay was developed for the simultaneous determination of nimesulide and of the identified metabolites in urine from treated subjects.

2. Experimental

2.1. Materials

Nimesulide (4-nitro-2-phenoxy-methanesulfonanilide) and hydroxynimesulide (metabolite M1, 2-[(4′-hydroxy)phenoxy]-4-nitro-methanesulfonanilide were kindly provided by Helsinn Healthcare SA (Switzerland). Metabolites M2, 4-amino-2-phenoxy-methanesulfonanilide, M3 2-[(4′-hydroxy)phenoxy] - 4 - amino - methanesulfonanilide, M4 4-*N*-acetylamino-2-phenoxy-methanesulfonanilide and M5 2-[(4′-hydroxy)phenoxy]-4-*N*-acetylamino-methanesulfonanilide were synthesized in our laboratories. HPLC-grade, analytical-grade organic solvents and Extrelut® cartridges (20 ml) were purchased from Merck (Bracco, Milan, Italy). HPLC-grade water was prepared with a Milli-Q water purification system. β-glucuronidase (5.5 U ml^{-1})/arylsulfatase (2.6 U ml^{-1}) was purchased from Boehringer Mannheim Italia (Monza, Milan, Italy).

2.2. Instrumentation

Electron impact (EI) mass spectra were obtained on a Shimadzu (Tokio, Japan) GC/MS QP1000 instrument (mass spectrometer quadrupole), equipped with a direct insertion probe (source temperature 200°C; electron energy 70 eV; software MSPAC 200).

LC analyses were carried out on a Hewlett Packard HP1050 Series (Milan, Italy) with a Supelcosil LC-18 DB column (25 cm × 4.6 mm i.d., particle size 5 μm) and a Supelgard™ LC-18 DB (20 × 4.6 mm i.d., particle size 5 μm). The HP1050 Series HPLC modules include a quaternary pump system, an autosampler, an UV/VIS Diode Array programmable detector, an online degasser and a HPLC^{3D} ChemStation (DOS series).

2.3. LC conditions

The HPLC pump was operated at a flow rate of 1.0 ml min^{-1} at room temperature (20 ± 2°C). The mobile phase was sodium phosphate buffer (50 mM) adjusted to pH 3 with *ortho*-phosphoric acid 85% (3.5 mM) (solvent A) and acetonitrile (solvent B); gradient elution was employed starting at 22% B, increasing linearly to 30% B over 18 min, then to 50% B over 50 min. Volumes of 20 μl were injected and the detector was set at 230 nm.

2.4. Treatment and urine collection

Six healthy volunteers (3 men and 3 women, 25–40 years old), which gave written informed consent to the study, were instructed not to take

any medication for 3 days prior to and during the study. The subjects fasted overnight and were orally given 200 mg nimesulide (2 sachets of 100 mg, Aulin, Boehringer Mannheim, Italia) and no food was allowed for at least 2 h nor alcoholic beverages at any time during the study. Urine was collected at 12, 24, 48, 72 and 96 h time intervals (blanks: urine before administration), measured and 20 ml aliquots of each sample were separately frozen at −20°C until analysis.

2.5. Extraction procedure and metabolites isolation.

Urine samples (20 ml) relative to the different observation times were centrifuged at 5,000 rpm and the supernatants were adjusted to pH 7.0 and extracted (Extrelut column) with ten volumes of dichloromethane containing 2.5% isopropanol. The organic extracts were concentrated in vacuo to dryness and the residues, taken up in acetonitrile (1 ml), were frozen at −20°C until quantitative analysis (free metabolites). Another 20 ml aliquot was adjusted to pH 4.5 with sodium acetate buffer (0.1 M) and submitted to enzymatic hydrolysis with β-glucuronidase/arylsulfatase (50 μl ml^{-1}; 36 h at 37°C); at the end of the incubation period the urine samples were adjusted to pH 7.0 with ammonium acetate (1 M) and then extracted as described (total metabolites). Conjugated metabolites were determined as difference between total and free metabolites.

For the characterization of nimesulide metabolites, the extracts from 0–48h and from 48–96 h unhydrolyzed and hydrolyzed urine samples were pooled for each subject and then submitted to bidimensional TLC analysis on Kieselgel GF254 nm plates (20 × 20 cm, Merck, Bracco, Milan, Italy). First run: toluene-ethylformiate-formic acid (50:40:10, v/v/v); second run: toluene-ethylacetate-isopropanol-ammonium hydroxyde 25% (30:30:30:10, v/v/v/v). The metabolites were visualized under an UV light (254 nm) and after spraying the plates with specific reagents for phenols (1:1 mixture of 2% ferric chloride in 2 N HCl and 1% aqueous potassium ferricyanide) and primary aromatic amines (1% sodium nitrite in 1N HCl followed by 0.2% β-naphtol in 1 N NaOH). The metabolites were extracted from silica gel plates with methanol (2 ml, 2 times) and the extracts, concentrated to small volume, subjected to EI mass spectrometry.

2.6. Standard solutions

Stock solutions (1 mg ml^{-1}) were prepared for nimesulide and each metabolite and stored frozen at −20°C. A mixed stock solution with a concentration of 10 μg ml^{-1} for each compound was prepared by dilution with acetonitrile. A serial dilution was made to give the mixed metabolite working solutions at concentrations 25, 50, 100, 250, 1000, 2500 and 5000 ng ml^{-1}. On the individual days of analysis, urine calibration standards containing 25, 50, 100, 250, 1000, 2500 and 5000 ng ml^{-1} of nimesulide and metabolites were prepared by adding 1 ml of the appropriate mixed working solution to 20 ml blank urine.

2.7. Assay validation

The method was validated over the concentration range 25–5000 ng ml^{-1} in human urine for nimesulide and each of its metabolites. The precision and accuracy were evaluated at the above concentrations over 3 days. In the HPLC precision, six replicates were analyzed at each concentration level on the same day, while in the between run analysis, duplicates were used at each concentration level. The method precision was calculated as the CV% of the daily averages.

3. Results and discussion

3.1. Urinary metabolic profile

TLC analysis of pooled extracts from the 0–48 h unhydrolyzed urine samples (subject 1) shows five metabolites (M1, M2, M3, M4 and M5): M5 the most abundant, M4 in trace amounts. The same metabolites were found in hydrolyzed urines, and a rough estimate of the spots size indicates that M1 and M5 metabolites are excreted mainly in conjugated form. The same metabolic profile was observed in all the remaining subjects.

In urine from later on (48–96 h), no additional metabolic species were found (only metabolite M5 in conjugated form) and no unchanged nimesulide was detected in any of the subject and at any observation time.

Fig. 1 reports the EI mass spectra of nimesulide and metabolites M1 and M2. The EI mass spectrum of nimesulide shows a $[M]^+$ at m/z 308 and a fragmentation pattern governed by cleavage of the sulfonamidic bond with formation of the ion at m/z 229, the base peak, from which originate the ions at m/z 199 $[229-NO]^+$ and 183 $[229-NO_2]^+$ due to the loss of nitro group and the ion at m/z 154 $[229-C_6H_4]^+$, from cleavage of the ether bond with retention of the oxygen on the nitrobenzene residue. Both the molecular ion, $[M]^+$ at m/z 324 and the main fragment (ion at m/z 245) are increased by 16 amu in M1, to indicate introduction of one oxygen atom in the molecule; the compound, on the basis of molecular weight and the fragmentation pattern, with typical losses of the nitro (ions at m/z 199 and 182) and phenol (ion at m/z 170) groups, was identified as 2-[(4′-hydroxy)phenoxy]-4-nitromethanesulfonanilide (hydroxynimesulide), the metabolite already identified. Metabolite M2 shows a $[M]^+$ at m/z 278, 30 amu less than that of the parent compound. The absence in the mass spectrum of the ions typical of nitro group and the poor fragmentation pattern support the structure of 4-amino-2-phenoxy-methanesulfonanilide, compound arising from reduction of the aromatic nitro group. The $[M]^+$ ion of metabolite M3 (Fig. 2) was at m/z 294, 16 amu over that of metabolite M2, indicating introduction of one oxygen atom: this was again on the aromatic nucleous, as confirmed by the presence of the ion at m/z 187 (loss of CO, typical of phenols, from the base peak at m/z 215); the fragmentation pattern is consistent with a structure of 2-[(4′-hydroxy)phenoxy]-4-amino-methanesulfonanilide, involving two different metabolic pathways, aromatic hydroxylation and nitro group reduction. Metabolite M4 (Fig. 2) arises from reduction of the nitro group and subsequent acetylation: this is well evidenced by the $[M]^+$ at m/z 320 and by some diagnostic ions due to the cleavage of the amidic bond: at m/z 278 $[M–CH_2=C=O]^+$, m/z 199 $[241–CH_2=C=O]^+$, and m/z 43 $[CH_3–CO]^+$ typical of *N*-acetylated derivatives. Both the molecular ion and the fragmentation pattern indicate for this compound the structure of 4-*N*-acetylamino-2-phenoxy-methanesulfonanilide. A similar fragmentation pattern was also observed in the EI mass spectrum (Fig. 2) of metabolite M5, one of the major metabolites in urine, with $[M]^+$ at m/z 336 and the main peaks at m/z 257 and 215, all of 16 amu over those of metabolite M4. The formation of compound, identified as 2-[(4′-hydroxy)phenoxy]-4-*N*-acetylamino-methanesulfonanilide, involves aromatic hydroxylation and nitro group reduction, this last followed by *N*-acetylation.

The structure of all the isolated metabolites was furtherly confirmed by comparing their chromatographic (TLC, HPLC) and mass spectrometric properties with those of the synthetic compounds.

3.2. Calibration

The calibration curves were constructed by weighted $1/x$ least-squares regression (8 points for each analyte) in blank urine samples from each subject in the concentration range 25–5000 ng ml^{-1} ($n=6$). For each compound the standard response was linear over the entire calibration range, with coefficients of determination greater than 0.9965 for nimesulide, 0.9994 for M1, 0.9989 for M2, 0.9972 for M3, 0.9991 for M4 and 0.9996 for M5. Fig. 3 shows a typical chromatogram obtained from an extract of blank urine spiked with 250 ng ml^{-1} of nimesulide and metabolites, showing base-line separation of all the compounds. The limit of detection (LOD) was estimated to be 10 ng ml^{-1} and the limit of quantification 15 ng ml^{-1}: this value was further validated by the analysis of 6 samples at this concentration value: precision was acceptable, since the %CV values did not exceed 15% for all examined compounds (data not shown).

3.3. Extraction efficiency and stability

A recovery test was performed by comparing the response of extracted samples spiked with nimesulide and metabolites before extraction with

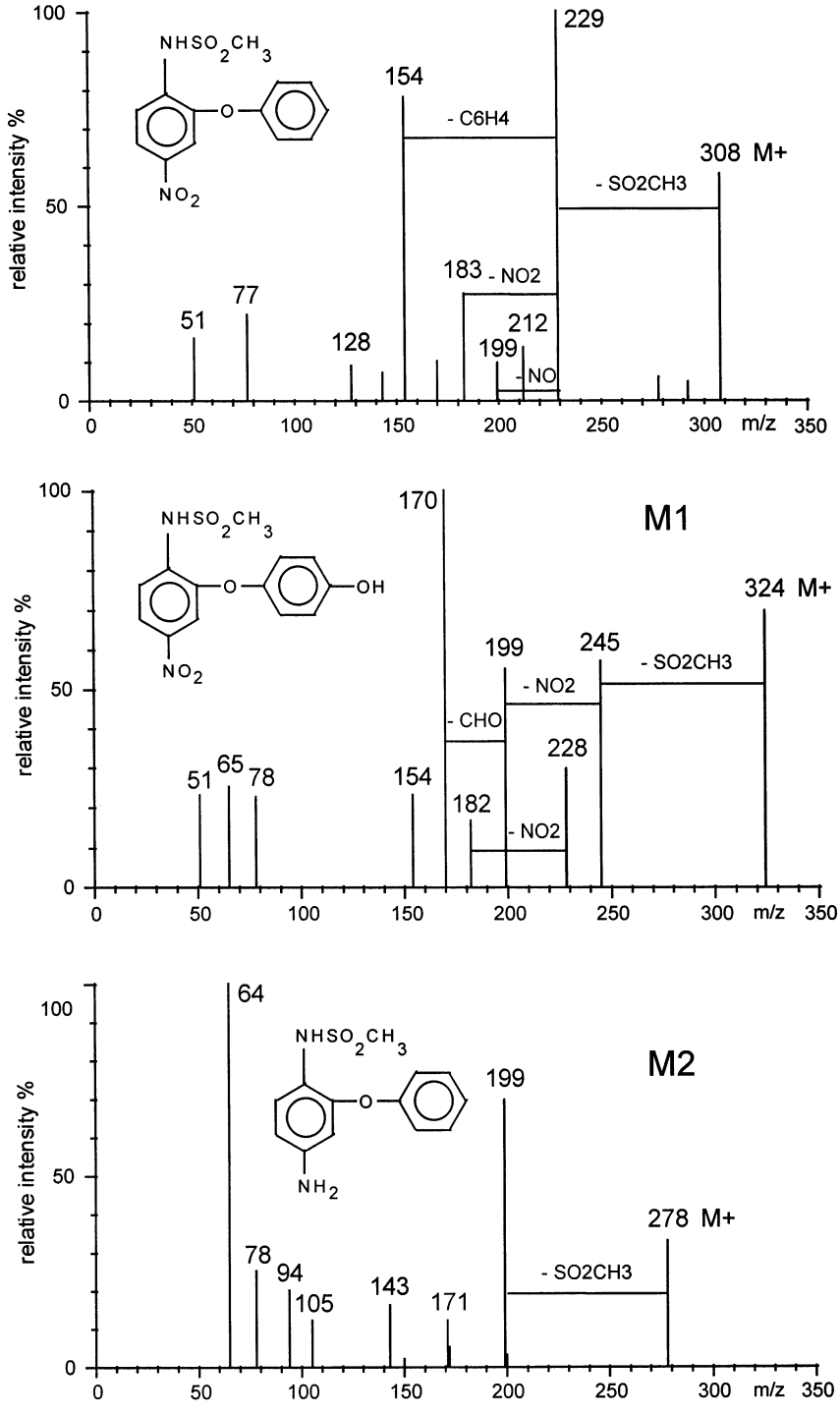

Fig. 1. Electron impact (EI) mass spectra of nimesulide and metabolites M1, M2.

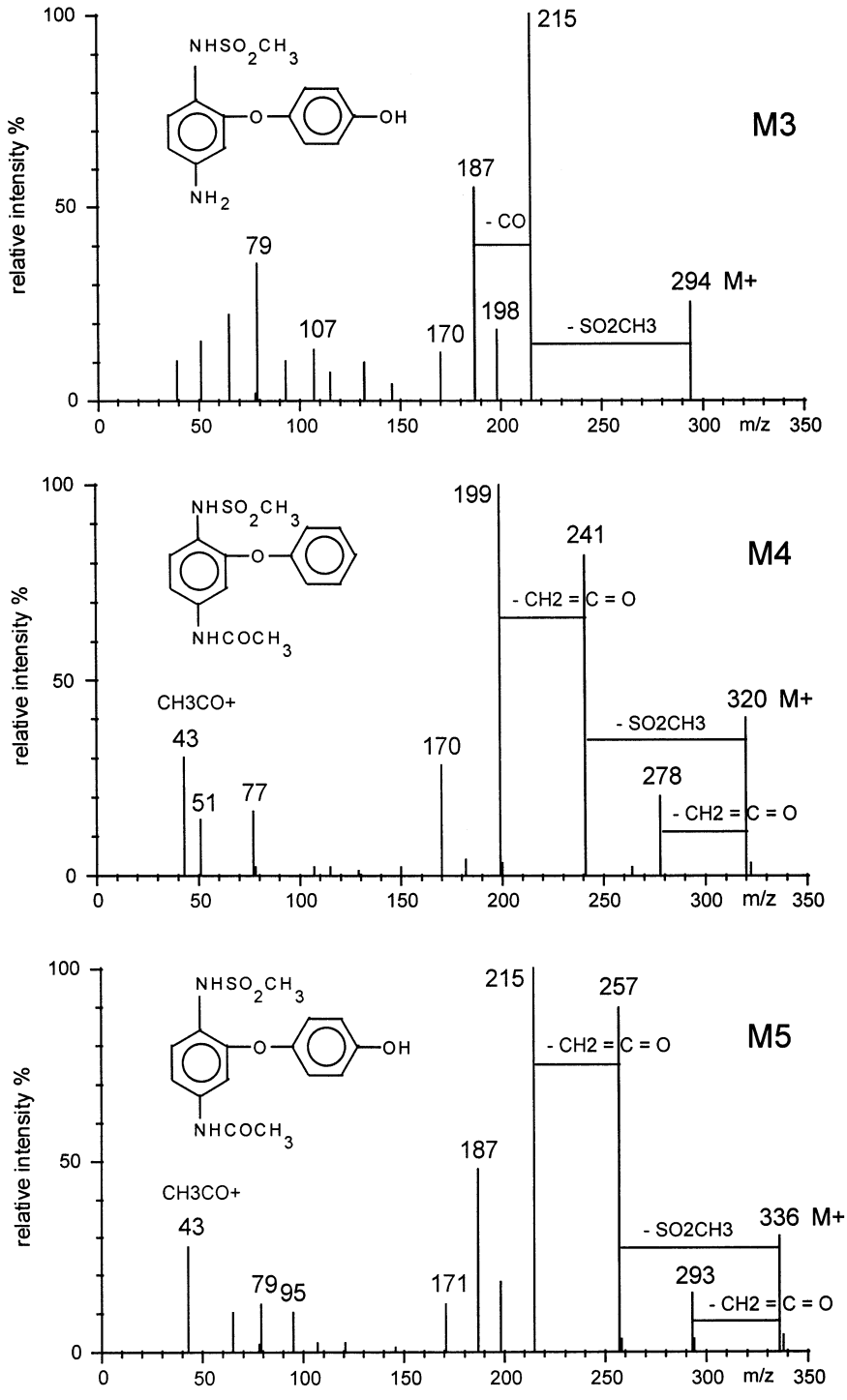

Fig. 2. Electron impact (EI) mass spectra of metabolites M3, M4 and M5.

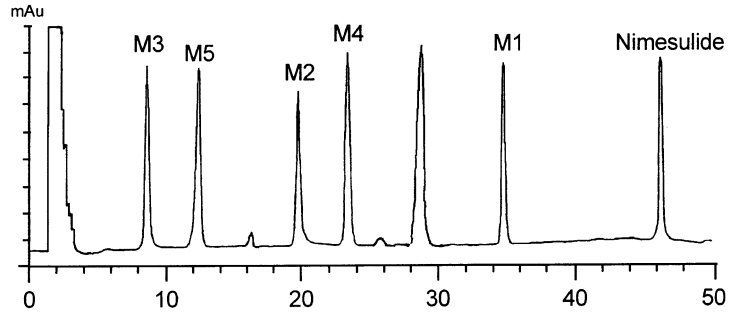

Fig. 3. Chromatogram of extracted blank human urine (unhydrolyzed) spiked with nimesulide and metabolites (250 ng ml^{-1} each).

that of extracted blank samples spiked just before injection (so as to compensate any effect due to the matrix). Percentage recoveries, determined at the two extremes of the calibration range ($n = 6$ each), were satisfactory, ranging from 91 to 99% for all of the compounds, except for metabolite M2, the averaged extraction efficiency being $\sim 80\%$ (Table 1).

Stability studies indicate that nimesulide and metabolites were stable throughout the sample storage and the chromatographic procedures. The recovery percentages of nimesulide and metabolites for the stability samples (blank urine spiked with two concentrations, 250 and 2500 ng ml^{-1} and extracts stored at -20°C; ten replicates at each level) were not statistically different from those obtained with freshly prepared extracts over a period of 6 months.

3.4. Precision and accuracy

The validation results for nimesulide and metabolites are shown in Table 2. The HPLC precision and accuracy of the method, determined at four concentration levels within the calibration range, was satisfactory, the coefficients of variance (CV%) for nimesulide and its metabolites ranging from 1.71 to 8.25% and %recovery from 91.8 to 107.2%. The same is for method precision and accuracy, the CV% relative to the four concentrations tested being between 1.18 and 6.73% and %recovery between 96.4 and 103.2%.

3.5. Urinary levels of nimesulide and metabolites

This method was applied to the analysis of urine samples from treated human subjects. In Fig. 4, typical chromatograms of unhydrolyzed and hydrolyzed urine samples taken at the 12–24 h interval from subject 2 are shown. Neither nimesulide nor metabolite M4 were detected, and all of the remaining metabolites, except for metabolite M2, were present in this subject both free and conjugated. The matrix does not interfere with the analysis of metabolites, since the blank components in both hydrolyzed and unhydrolyzed samples were eluted within the first minutes of the chromatographic run or sharply separated from the metabolites (obviously the purity of the peaks of metabolites was always checked during analysis).

Table 1
Extraction recoveries of nimesulide and metabolites from human urine

Compound	Concentration (ng ml^{-1})	Recovery (%)
Nimesulide	25	96.45 ± 3.21
	5000	96.22 ± 2.09
M1	25	99.32 ± 2.80
	5000	98.56 ± 2.08
M2	25	79.43 ± 2.52
	5000	78.89 ± 1.46
M3	25	98.75 ± 3.30
	5000	96.77 ± 1.83
M4	25	93.80 ± 2.72
	5000	92.74 ± 1.30
M5	25	91.13 ± 3.19
	5000	91.78 ± 1.31

Means $\pm$ SD of six determinations.

Table 2
Assay validation

Compounds	Nominal (ng ml^{-1})	Intra-assay[a]		Inter-assay[b]	
		HPLC precision[c]	Accuracy[d]	Method precision	Accuracy
Nimesulide	25	5.21	92.5	6.73	96.4
	50	3.14	101.8	4.82	98.7
	2500	4.02	97.4	5.21	102.4
	5000	2.21	105.0	1.80	101.8
M1	25	6.70	97.2	4.08	97.5
	50	3.20	107.2	4.75	102.5
	2500	1.75	98.7	3.04	101.4
	5000	1.80	104.3	2.15	98.4
M2	25	8.25	93.6	5.75	98.6
	50	3.13	104.3	3.24	103.2
	2500	1.80	101.5	2.21	100.8
	5000	2.09	96.9	1.18	98.2
M3	25	5.25	103.5	4.21	101.4
	50	3.69	101.2	2.65	99.7
	2500	4.37	96.8	3.12	98.5
	5000	2.18	98.2	1.95	101.6
M4	25	7.21	106.5	5.21	102.7
	50	3.85	95.7	4.02	98.5
	2500	3.12	96.8	3.85	97.4
	5000	1.71	102.4	2.02	100.8
M5	25	4.92	91.8	3.12	96.7
	50	4.01	95.8	2.57	98.4
	2500	5.02	105.4	4.21	102.5
	5000	2.56	102.6	2.97	99.4

[a] Six replicates at each level.
[b] Six runs, duplicates at each level in each run.
[c] Coefficient of variance in percentage (CV%).
[d] Mean recovery percentage.

The results relative to the quantitative determination of nimesulide and its urinary metabolites are reported in Table 3, where values are expressed as a percentage of the administered dose and as a mean of six subjects. The excretion of unchanged drug in the 96 h interval is negligible ($< 0.5\%$, data not shown). In all subjects, it was below the limit of detection or quantification, and the same is for metabolite M4, which was detectable, in two subjects only, and at the first observation times.

The percentage of excretion of the drug in metabolized form in the overall 96 h interval (only conjugated M5 was still detectable between 72 and 96 h) accounts for 40% of the administered dose, approximately equally distributed between M1 ($17.55 \pm 3.6\%$) and M5 ($19.07 \pm 4.3\%$), which are both excreted mainly in conjugated form. The percentage of excretion of metabolite M2 is minimal ($0.72 \pm 0.4\%$ within the first 24 h), and that of metabolite M3, both in free and conjugated form, does not exceed 3% of the dose. The bulk of metabolite excretion is within the first 24 h (28.43% of the dose). Also, while M1 excretion peaks at 12 h, the appearance of metabolite M5, which arises from four different metabolic steps (hydroxylation, nitro group reduction, *N*-acetylation and finally conjugation), is delayed (2.11% at the 12th h): it peaks in the 12–24 h interval, it is the main metabolite in the 24–48 h and only present in all subjects in the remaining 48–96 h. Although the observation is limited to a small number of subjects, no significant differences were observed in the quantitative metabolic profile of the drug between male and female subjects.

Table 3
Urinary levels of nimesulide metabolites in man ($n = 6$) following administration of a single, oral dose of 200 mg nimesulide

Compound	0–12 h		12–24 h		24–48 h		48–72 h		72–96 h		Total % of dose
	F	C	F	C	F	C	F	C	F	C	
M1	0.02 (0.005)	8.93 (2.73)	0.01 (0.008)	7.45 (3.15)	—	1.14 (0.85)	—	—	—	—	17.55 ± 3.6
M2	0.43 (0.25)	0.06 (0.07)	0.12 (0.11)	0.11 (0.08)	—	—	—	—	—	—	0.72 ± 0.43
M3	0.06 (0.08)	0.42 (0.25)	0.16 (0.08)	1.27 (0.75)	0.09 (0.06)	0.45 (0.37)	—	—	—	—	2.45 ± 1.22
M5	0.15 (0.12)	1.96 (1.33)	0.43 (0.35)	6.85 (2.94)	0.38 (0.24)	4.73 (2.28)	0.2 (0.15)	3.12 (1.28)	—	1.25 (0.87)	19.07 ± 4.3
Total % F+C	12.03		16.40		6.79		3.32		1.25		39.79 ± 3.7

F, free metabolites; C, conjugated metabolites.
In brackets ± S.D. relative to six subjects.

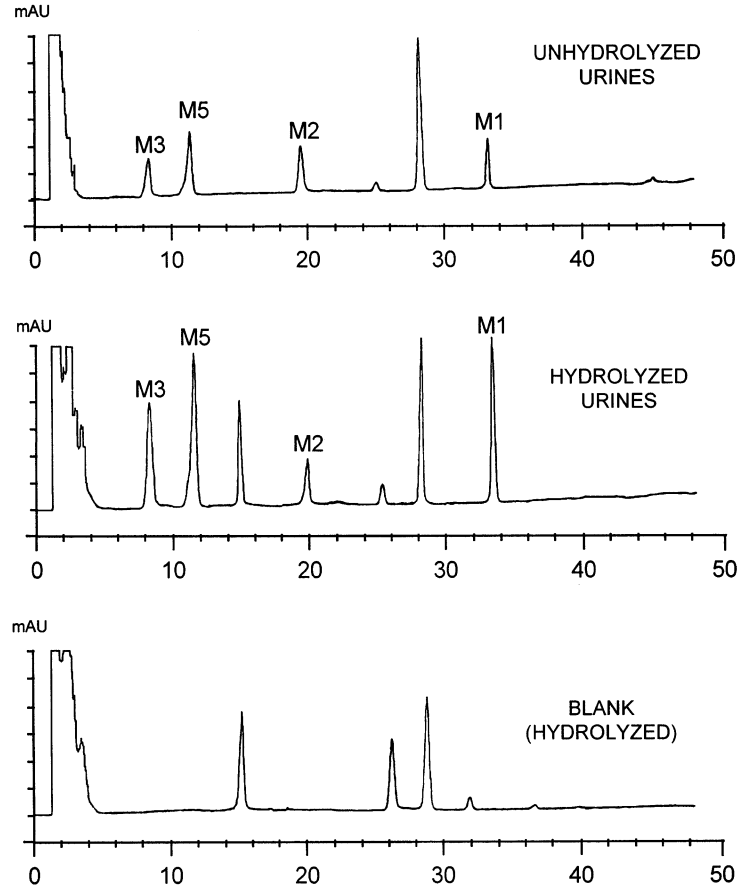

Fig. 4. Chromatograms of human urine extracts before and after enzymatic hydrolysis following an oral dose of 200 mg nimesulide (12–24 h interval; subject 2).

4. Conclusions

The results of this study show that when the non steroidal anti-inflammatory drug nimesulide is orally given, it undergoes hepatic metabolization to five main species which have been unequivocally characterized by means of mass spectrometric analysis and further chemical synthesis as the products of aromatic oxidation, nitro group reduction, *N*-acetylation and conjugation. It is not the aim of the present paper to draw a mechanistic study of their formation, however, on the basis of a grossly examination of their appearance along the time and of previous in vitro evidences in the rat (where the qualitative metabolic profile is the same as in man), it seems that the oxidative attack of the molecule plays a key role in its elimination [3].

A new and sensitive HPLC assay was developed and validated for the simultaneous determination of nimesulide and all its metabolites in human urine and its utility has been demonstrated in experiments following oral administration of 200 mg of the drug. The method, based on a reverse-phase gradient elution approach, allows with a single chromatographic run, the sharp separation of the unchanged drug and all its metabolites. Several reverse-phase columns were tested to optimize the separation procedure, and the double bonded LC-18 column was found to be the more effective for this purpose and for removal of matrix interferences. When dealing

with a mixture of six analytes, the chromatographic conditions were markedly modified in respect to those previously developed by Castoldi et al. [2] which report the separation of nimesulide and only one metabolite (hydroxynimesulide) by isocratic elution.

In the absence of quantitative data on faecal excretion, we can not establish a mass balance of nimesulide excretion. However, the total urinary excretion in the 96th hour, $\sim 40\%$ of the dose, is lower than that previousy reported in a dose-balance study in man [4] involving the administration of 100 mg ^{14}C labelled nimesulide, where 78.1% of the radiolabelled dose was recovered (60.2% in urine and 17.9% in faeces).

The lower recovery in urines may indicate: (1) an incomplete gastrointestinal absorption of the drug at the dosage employed; (2) a consistent faecal excretion of the metabolites. This is reasonable since the conjugative pathway plays a key role in their elimination in urine, and a large amount of conjugated species might be transported into the bile and excreted in faeces as such or after intestinal deconjugation. Both of these possibilities have been confirmed, although qualitatively, through the analysis of faecal samples (24 and 48 h) from three subjects, which indicates the presence of unchanged nimesulide and of metabolites M2, M3 and M5, in free and conjugated form; (3) Finally, the existence of other metabolic species in urine not extractable/detectable in the described conditions. A metabolite from an oxidative O-dearalchylation process has been excluded, since we never found the corresponding phenolic metabolite, 4-nitro-2-hydroxy-methanesulfonanilide [5]. On the other hand, a metabolic step involving $NO_2/NHSO_2CH_3$ loss and subsequent conjugation with glutathione, with the formation of highly polar metabolites, can not be excluded as a possible route of nimesulide elimination.

References

[1] R. Davis, R.N. Brogden, Drugs 48 (1994) 431–454.
[2] D. Castoldi et al. J. Chromatogr. 425 (1988) 413–418.
[3] R. Maffei Facino, M. Carini, R. Stefani, C. Marinello, A. Macciocchi, Pol. J. Pharmacol. 46 (1994) 357–358.
[4] A. Bernareggi, Drugs 46 (1) (1993) 64–72.
[5] R. Maffei Facino, M. Carini, G. Aldini, Drugs 46 (1) (1993) 15–21.