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Simultaneous determination of nimesulide and hydroxynimesulide in rat plasma, cerebrospinal fluid and brain by liquid chromatography using solid-phase extraction

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Abstract

A liquid chromatographic method with UV detection for the quantification of nimesulide (N) and hydroxynimesulide (M1) in rat plasma, cerebrospinal fluid (CSF) and brain tissue is reported. Plasma samples (250 μl) and brain homogenates added with the right amount of the internal standard (I.S., 2′-(cyclohexyloxy)-4′-nitrophenyl methanesulphonanilide, NS398) are extracted on C_{18} disposable cartridges by solid-phase extraction (SPE), while CSF samples are analyzed without any extraction. The separation is performed at room temperature on a Waters Symmetry C_{18} 3.5 μm (150×4.6 mm I.D.) column with acetonitrile–sodium citrate buffer pH 3.00 (53:47, v/v) as mobile phase, at a flow-rate of 1.1 ml/min and detection at 240 nm. The retention times are 3.3, 6.0 and 9.9 min for M1, N and I.S., respectively. The lower limits of quantitation for either nimesulide and M1 are 25 ng/ml for plasma, 20 ng/ml for CSF and 25 ng/g for brain tissue. The calibration curves are linear up to 10 000 ng/ml for plasma, 5000 ng/ml for CSF and 5000 ng/g for brain tissue. This new assay can be applied to the study of the role of nimesulide in the modulation of neuroinflammatory processes.

Keywords: Nimesulide; Hydroxynimesulide

1. Introduction

Nimesulide, 4′-nitro-2′-phenoxy-methanesulphonanilide (Fig. 1A), is a non-steroidal anti-inflammatory drug (NSAID) that, at therapeutic doses, markedly inhibits cyclooxygenase (COX)-2 with less effect on COX-1 [1]. Numerous studies have demonstrated the good antipyretic, analgesic and anti-inflammatory activities of N in a wide range of clinical conditions [2]. In the last few years it has been demonstrated that prostaglandins (PGs), which are synthesized from arachidonic acid by COX, play an important role in the modulation of spinal neuron activity and that NSAIDs have a direct action on central processing of peripheral information [3,4]. Furthermore, on the light of epidemiological findings, some investigators have recently suggested a potential utility of NSAIDs for the treatment of different disorders such as cancer and dementia [5,6]. In particular, the selective COX-2 inhibitors slow the progression of dementia in Alzheimer patients [7,8]. For all these reasons, the opportunity to determine N

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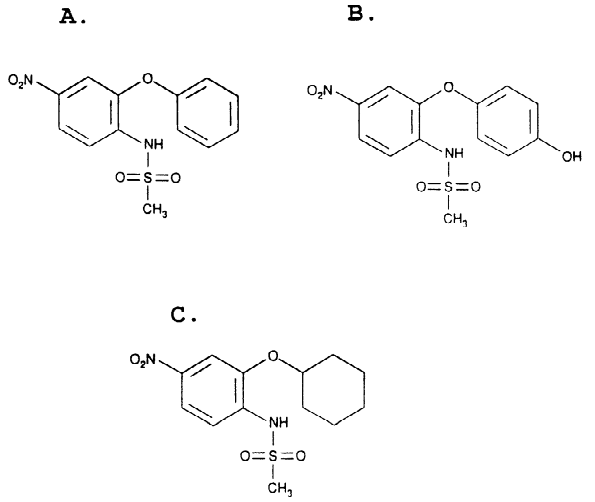

Fig. 1. Structural formulae of N (A), M1(B) and I.S. (C).

in plasma and in the central nervous system at the same time is of crucial importance to better evaluating the effects of the drug and to understanding the pharmacokinetic–pharmacodynamic relationship in the inhibition of neuroinflammation exerted by this drug.

The measurement of N plasma levels in humans and animals has been previously carried out by high-performance liquid chromatography (HPLC) [9–14], high-performance thin-layer chromatography (HPTLC) [15] and HPLC tandem mass spectrometry [16]. The simultaneous determination of N and its major metabolite 2-[(4′-hydroxy)phenoxy]-4-nitromethanesulfonanilide (M1, Fig. 1B) in human plasma and urine was performed by means of LC [17–19]. Apart for one of them [13], all these methods used single or repeated liquid–liquid extraction of the sample and therefore are clearly time consuming. Moreover, no methods have been developed so far for the determination of N and its metabolite in the central nervous system.

The aim of the present work was to develop a simple, rapid and sensitive assay, using a solid-phase extraction (SPE) procedure that allows the simultaneous determination of N and its M1 metabolite in plasma, CSF and brain of rats treated with an anti-inflammatory dose of N (5.0 mg/kg p.o.).

2. Experimental

2.1. Chemicals and materials

N and M1 were kindly provided by Helsinn Healthcare (Pambio-Noranco, Switzerland). Internal standard, 2′-(cyclohexyloxy)-4′-nitrophenyl methanesulphonanilide NS398 (Fig. 1C), was purchased from Inalco (Milan, Italy) while disodium orthophosphate dodecahydrate, sodium citrate and orthophosphoric acid, all of analytical grade, were obtained from Sigma (St. Louis, MO, USA). Methanol and acetonitrile (LC grade) were purchased from Merck (Darmstadt, Germany). Heparin was obtained from Parke-Davis (Milan, Italy).

Disposable C_{18} 100 mg/1 ml cartridges for SPE were produced by International Sorbent Technology (Hengoed, Mid Glamorgan, UK).

2.2. HPLC system and chromatographic conditions

A Millipore Waters Model 590 liquid chromatograph (Waters, Milford, MA, USA) with a variable wavelength LC75 Spectrophotometric UV detector Perkin-Elmer (Norwalk, CT, USA) was employed. The injection valve was a model 7125 Rheodyne (Cotati, CA, USA). The system was connected to a D-2000 chromato-integrator Hitachi–Merck (Merck). A Waters Symmetry C_{18} 3.5 μm (150×4.6 mm I.D.) coupled to a Waters Sentry Symmetry C_{18} guard column was operated at room temperature. The mobile phase was acetonitrile–50 m*M* sodium citrate buffer, pH 3.00 (53:47). The buffer was prepared fresh dissolving 16.8 g of citric acid and 2.66 g of NaOH in 1000 ml of bidistilled water and adjusting the solution to pH 3.00 with orthophosphoric acid. Before mixing with acetonitrile the buffer solution was filtered through a cellulose filter (0.45 μm HV Millipore). The flow-rate was 1.1 ml/min and the elution was monitored at 240 nm.

2.3. Animals and treatment

Male virus-free Swiss-derived albino Crl:CD (SD) Br rats (250–350 g b.w.) were purchased by Charles River (Calco, Italy). The animals were housed four per cage in Mackolon cages (21 °C temperature,

50–60% relative humidity and 12 h light–dark cycle) and fed on a commercial pellet diet.

N was suspended in 0.9% NaCl and administered orally at a dose of 5.0 mg/kg.

2.4. Sample collection

All samples were collected 60 min after drug administration. Under barbiturate anaesthesia (Nembutal 50 mg/kg i.p.), CSF was collected into ice-cold Eppendorf microvials by cisternal puncture performed with a 26G needle attached to a PE 10 cannula and then immediately frozen. Afterwards the animals were sacrificed by decapitation and blood was collected in heparinized polypropylene tubes (containing 45 USP units sodium heparin) and centrifuged at 2500 *g* for 10 min. Plasma was transferred in polypropylene tubes and immediately frozen. Brain was rapidly removed, deprived of cerebellum, then washed with ice-cold 0.9% (w/v) saline, dried with tissue paper, weighed and rapidly frozen on dry ice.

All frozen plasma, CSF and brain samples were stored at −20 °C until analysis.

2.5. Sample preparation

CSF samples (200 μl) were thawed at room temperature for about 10 min and vortexed for 30 s; then 100 μl were directly injected into the chromatographic system without any extraction procedure. Therefore, no I.S. addition was necessary.

Frozen plasma samples were thawed at room temperature for about 30 min then vortexed for 30 s. Plasma (250 μl) was pipetted into a 5-ml polypropylene tube and addition of 1 ml of ice-cold 50 m*M* $Na_2HPO_4 \cdot 12H_2O$, pH 3.00 buffer, containing 2500 ng of the I.S. This mixture was vortexed for 30 s, then extracted.

Frozen brain samples were transferred in 10-ml polypropylene centrifuge tubes. After the addition of ice-cold 50 m*M* $Na_2HPO_4 \cdot 12H_2O$, pH 3.00 (5 ml/g) containing the I.S. (250 ng/ml), the tissue was homogenized with a Polytron and centrifuged at 9000 *g* for 10 min in a Sorvall RC-5 refrigerated centrifuge. The supernatant was transferred into another tube and an aliquot of 2 ml was extracted.

2.6. SPE procedure

Solid-phase extraction of plasma and brain samples was performed on disposable C_{18} cartridges primed with 1 volume of methanol and 1 volume of 50 m*M* $Na_2HPO_4 \cdot 12H_2O$, pH 3.00 buffer. Plasma or brain homogenate, prepared as described above, were loaded on the conditioned cartridges. After washing with two volumes of the same phosphate buffer, the elution was carried out with two fractions of 0.5 ml of methanol. The eluate was evaporated to dryness at 40 °C under nitrogen flow, and the residues reconstituted in 100 μl of mobile phase. Aliquots (50 μl) of this solution were injected into the HPLC system.

2.7. Calibration curves and method validation

The validation of this analytical method was performed according to the requirements indicated by Lindner and Wainer [20] and the procedure described by Hartman et al. [21].

The linearity was assessed in the range of 25–10000 ng/ml for plasma, 25–5000 ng/g for brain tissue and 20–5000 ng/ml for CSF. The calibration curves were constructed for rat plasma or brain with animal blank plasma or brain homogenates containing added concentrations (25, 100, 500, 2000, 10 000 ng/ml for plasma, and 25, 100, 500, 2000, 5000 ng/g for brain tissue) of N and M1. Calibration curves were drawn for N and M1 concentration against the peak-area ratio of N and M1 to I.S., applying least-squares linear regression analysis, by using the Sigma Stat 5 computer program (SPSS Science, Chicago IL, USA).

CSF standard samples were prepared adding N and M1 at concentrations of 20, 100, 500, 2000, 5000 ng/ml to blank rat CSF and analysed without any extraction. The calibration curves were obtained by plotting the peak area versus N and M1 concentration (ng/ml), as described above. Five identical samples for each curve point were analysed for the assessment of calibration curves and for the determination of the limit of quantitation.

The lower limit of quantitation (LLOQ) was estimated in the process of calibration curve construction and 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%.

Pooled rat ''blank'' plasma and brain homogenate samples as well as rat CSF ''blank'' samples were examined to confirm the absence of interfering peaks.

Extraction recovery was determined in the following way. A plasma or brain homogenate sample spiked with N and M1 was extracted; the SPE cartridge eluate was collected into a vial containing a known amount of I.S. and then processed and analysed as described above. The non-extracted samples were prepared by mixing solutions containing the same amount of the compounds spiked in plasma and brain homogenate. After evaporation to dryness, the residue was reconstituted with mobile phase (100 μl) and analysed as described above. The percentage recovery from plasma and brain tissue were calculated by comparing peak–area ratios obtained with the above mentioned procedure (extracted versus non-extracted). The same approach was used for the determination of the recovery of the I.S., except that N was used as the ''internal standard''. Recovery was assessed by repeating three times the analysis of plasma or brain samples containing 100, 2000, 8000 ng/ml (plasma) and 100, 2000, 5000 ng/g (brain) of N and M1.

The stability of the analyte was investigated as follows. A set of standard samples was made from a freshly prepared stock solution of the analyte in drug-free plasma, CSF and brain homogenate. Three aliquots at both low and high concentrations (50 and 5000 ng/ml for plasma and CSF or 50–5000 ng/mg for brain) were prepared for each study and kept frozen at −20 °C. Short-term stability was tested analysing the samples thawed and left 4 h at ambient temperature on the bench. For freeze–thaw stability study, the samples were left 1 h on the bench to thaw unassisted, then refrozen for 24 h. This cycle was repeated three times and analysis was done after the third freeze–thaw cycle. Long-term stability study was performed analysing the samples stored for 3 months at −20 °C. The stability of the analyte in the final extract from plasma and brain tissue was tested performing a second analysis of the same extract left 24 h at room temperature.

3. Results

The chromatograms obtained from drug-free extracts of plasma and brain tissue as well as the non-extracted blank CSF are shown in Figs. 2A, 3A and 4A. To obtain a satisfactory separation with a good resolution of the peaks, several mobile phases and columns were tried and the best results are given in Figs. 2B, 3B and 4B. In all matrices the employed chromatographic conditions produced sharp and symmetrical peaks for M1, N and I.S. with convenient retention times (3.3, 6.0 and 9.9 min, respectively). Although near to the M1 peak another peak was observed, this latter did not influence the quantitative evaluation of M1, being the peaks well resolved. The chromatograms obtained from plasma and brain extracts as well as from the non-extracted CSF samples of rats treated with N show no other peak interfering with any of the tested compounds (Figs. 2C, 3C and 4C). The chromatograms were reproducible for about 200 injections of CSF or plasma extracts or 100 injections of brain tissue extracts. Afterwards, to keep the reproducibility a new analytical column with a new guard column should be used.

The calibration curves were found to be linear for both compounds over the range 25–10 000 ng/ml for plasma, 25–5000 ng/g for brain tissue and 20–5000 ng/ml for CSF. The results of linear regression analysis of the calibration curves generated during the course of the study are shown in Table 1. The mean correlation coefficients for both N and M1 were >0.99 in plasma and brain tissue and >0.999 in CSF. The limit of quantitation for either N and M1 was 25 ng/ml in plasma, 25 ng/g in brain tissue and 20 ng/ml in CSF, with both precision (RSD %) and accuracy values $\leq 15\%$. The intra- and inter-days precision and accuracy during the validation study were within acceptable limits (Tables 2 and 3).

Different solid-phase extraction cartridges (C_2, C_8, C_{18}) and two extraction buffers at pH 3.00 and pH 7.00 were tested. We selected C_{18} cartridges and the pH 3.00 buffer because they allowed the highest extraction recovery of the drug and M1 from plasma and brain tissue under the assay conditions. N, M1 and I.S. were almost fully recovered from plasma and brain tissue, as demonstrated by the recovery values given in Table 4.

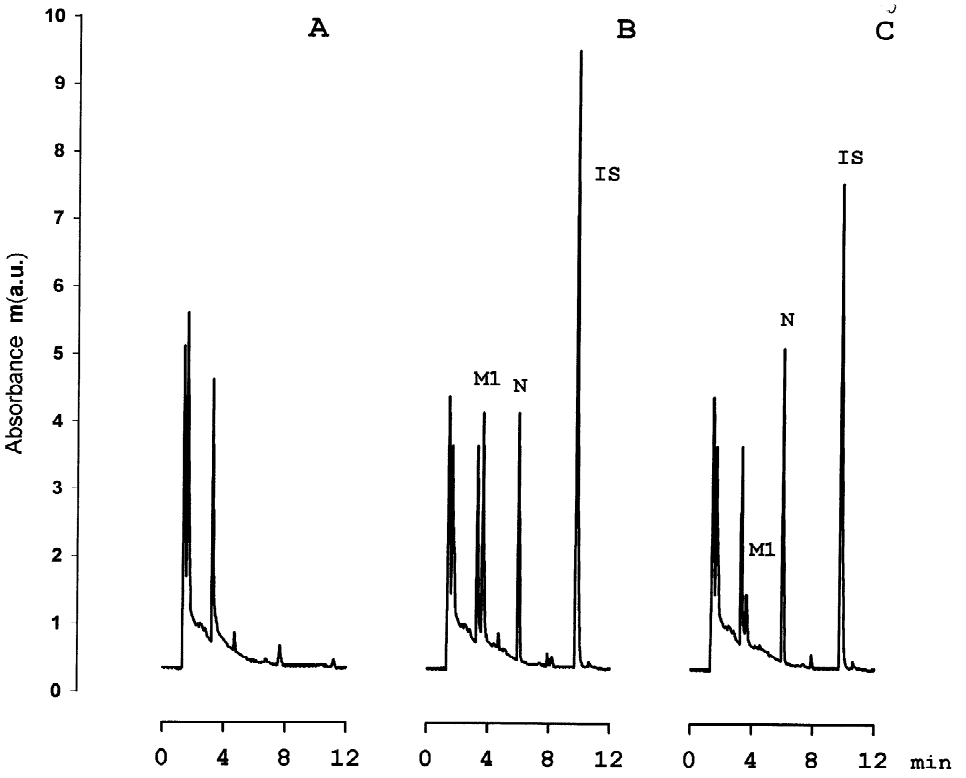

Fig. 2. Chromatograms of extracted sample of blank rat plasma (A), of rat plasma spiked with 500 ng/ml of N and M1 (B) and of plasma obtained 1 h after the oral administration of 5 mg/kg nimesulide containing 815.5 ng/ml of N and 141.1 ng/ml of M1 (C).

In Table 5 are summarized the results of the stability study. N and M1 were found to be stable in rat plasma, brain tissue and CSF samples for at least 12 weeks when stored at −20 °C and for 1 week when subjected to three freeze–thaw cycles. N and M1 in plasma and brain tissue extracts were also stable for at least 24 h when left at room temperature.

4. Discussion

We present here a new, efficient and accurate method for the extraction and quantification of N and M1 in plasma and central nervous system of animals treated with an anti-inflammatory and anti-hyperalgesic dose of N [22].

To the best of our knowledge, this method is the first to allow a simultaneous determination of N and its main metabolite in plasma and in the central nervous system. In particular, although different methods for the measurement of N and M1 in plasma have been recently published [9–18], we are unaware of any report on LC methods for measuring their concentrations in the CSF and in the brain. This opportunity seems to be of particular importance in the light of the recent data concerning the ability of NSAIDs to act in the central nervous system and of the possible clinical significance of these observations [6–8]. With regard to the technical aspects of our work, it is important to note that the use of solid-phase extraction offers several significant advantages, which include rapid sample processing, reduced organic solvent use (with consequently reduced exposure of analyst to toxic substances) and more reproducible results. With regard to plasma samples, our extraction method is simpler and less time-consuming than others previously reported in the literature in which solvents such as toluene, benzene, dichloromethane or chloroform were used

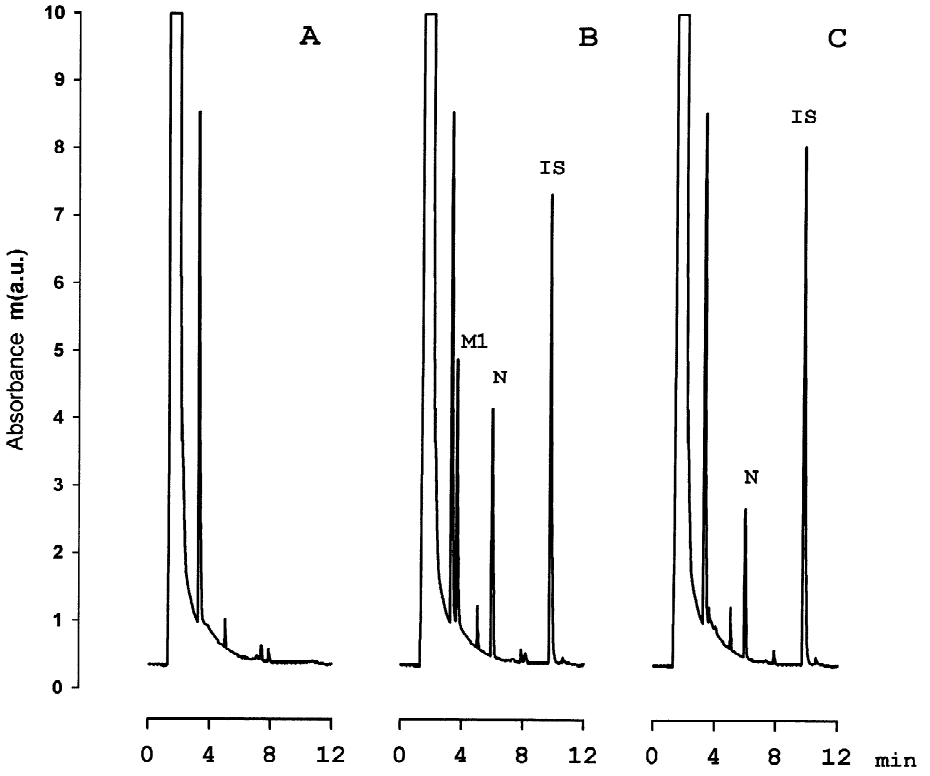

Fig. 3. Chromatograms of extracted sample of blank rat brain tissue (A), of brain tissue spiked with 500 ng/g of N and M1 (B) and of brain tissue obtained 1 h after the oral administration of 5 mg/kg nimesulide, containing 270.6 ng/g of N (C).

and single or repeated liquid–liquid extraction of the sample were employed. Moreover, the use of an I.S. such as NS398 which is a structurally related analogue of N, allowed us to apply the same method for the extraction of N and M1 from plasma and brain tissue.

It has been demonstrated that depending on the pH of the medium N is found in two different forms, ionised and non-ionised, due to its pK_a=6.5 [23,24]. Thus the pH of extraction buffer and mobile phase is very important in the determination of N. Near to pH 3.00 N is present almost completely in the non-ionised form which is easily retained by the C_{18} stationary phase of the extraction column. Therefore, this pH value should be considered as an optimal condition for the drug extraction. Similarly, to obtain a good separation of N, M1 and I.S. with the selected column, we had to employ an acidic mobile phase. In this condition, the detection wavelength of 240 nm allowed us to reach a very satisfactory LLOQ. Other authors used a more selective wavelength (404 nm) at higher pH to measure the human plasma levels of N alone, without using an I.S. [13]. At this wavelength (404 nm) the absorbance of the three compounds, with particular reference to I.S., in acidic ambient decreased dramatically [24]. For these reasons, we preferred to use a wavelength of 240 nm.

The same chromatographic conditions we used for the determination of N and M1 in plasma and brain allowed us to measure their lower concentrations in CSF samples without using any extraction procedure nor I.S. The LOQ of N and M1 in CSF (4 ng/200 μl) depends also by the fact that the whole volume of each sample (200 μl) can be directly injected into the chromatographic system.

Another thing we should stress about the present method is the short analysis time (<11 min), which permits multiple determinations per hour. The small amount of the sample required for the measurement, as well as the sensitivity comparable to the HPLC

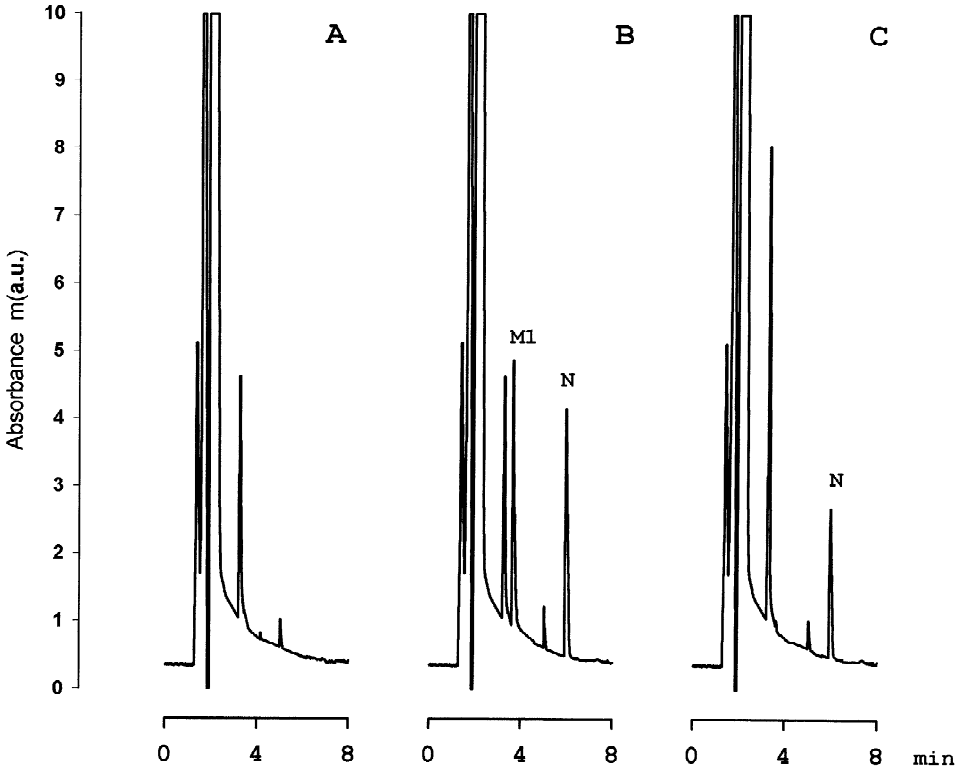

Fig. 4. Chromatograms of blank rat CSF (A), of rat CSF spiked with 250 ng/100 μl of N and M1 (B) and of rats CSF sample obtained 1 h after the oral administration of 5 mg/kg nimesulide, containing 300 ng/ml of N (C).

tandem mass spectrometry method [16] make possible the evaluation of very low N and M1 levels in biological fluids and tissue.

In this study we measured the concentrations of N and of its major metabolite in the plasma and central nervous system of animals treated by oral route with a single, pharmacologically active, dose of N. On the basis of the present data, this method is the best and simplest yet available allowing simultaneous quantification of N and its major metabolite not only in

Table 1
Linear regression analysis of calibration curves of N and M1 (n=5)

		Mean	SD	RSD
N in plasma	Slope	9.453	0.108	1.14
	Intercept	−0.01	0.024	–
M1 in plasma	Slope	9.338	0.162	1.73
	Intercept	0.005	0.03	–
N in brain tissue	Slope	0.0786	0.00163	2.07
	Intercept	0.0046	0.0054	–
M1 in brain tissue	Slope	0.0761	0.00234	3.07
	Intercept	0.0042	0.0058	–
N in CSF	Slope	0.000627	0.00000952	1.52
	Intercept	−5.422	1.018	–
M1 in CSF	Slope	0.000616	0.0000173	2.81
	Intercept	0.384	1.417	

Table 2
Intra day precision and accuracy of the analysis of N and M1 in spiked rat plasma, brain and CSF

Sample	N					M1			
	Added amount	Found (mean)	SD	Precision RSD% (n=5)	Accuracy % (n=5)	Found (mean)	SD	Precision RSD% (n=5)	Accuracy % (n=5)
Plasma	25	23.8	1.3	5.5	95.2	26.0	1.4	5.6	104.0
ng/ml	100	95.4	5	5.2	95.4	96.6	5.2	5.2	96.6
	500	484	23	4.8	96.8	487	16.3	3.3	97.4
	2000	1944	58	3.0	97.2	1964	67	3.4	98.2
	10 000	9830	270	2.7	98.3	9874	281	2.8	98.7
CSF	20	20.7	0.8	3.9	103.5	20.5	1.1	5.5	102.5
ng/ml	100	98.7	2.8	2.8	98.7	101.0	3.2	3.2	101.0
	500	496	9.9	2.0	99.2	497	12.3	2.5	99.4
	2000	1912	51	2.7	95.6	1958	47	2.4	97.9
	5000	4963	83	1.7	99.3	4976	91	1.8	99.5
Brain	25	22.4	2.4	10.7	89.6	22.7	2.1	8.4	90.8
ng/g	100	90.2	4.7	5.2	90.2	96.2	4.9	4.9	96.2
	500	463	22.9	4.9	92.6	471	23.8	4.8	94.2
	2000	1884	124	6.6	94.2	1906	82	4.1	95.3
	5000	4610	310	6.7	92.2	4756	298	6.0	95.1

plasma but also in CSF and brain tissue. Therefore, we think it might be suitable to assess the pharmacokinetic–pharmacodynamic relationship of this drug not only in periphery but also in the central nervous system. Such an information will surely allow to a more rational evaluation and use of N as a drug for the treatment of inflammatory pain and neuroinflammatory diseases.

Table 3
Inter-day precision and accuracy of the analysis of N and M1 in spiked rat plasma, CSF and brain

Sample	N					M1			
	Added amount	Found (mean)	SD	Precision RSD% (n=5)	Accuracy % (n=5)	Found (mean)	SD	Precision RSD% (n=5)	Accuracy % (n=5)
Plasma	25	23.6	1.6	6.8	94.4	25.8	1.8	7.0	103.2
ng/ml	100	96.5	6.1	6.3	96.5	95.4	5.6	5.9	95.4
	500	459	28	6.1	91.8	473	18.7	4.0	94.6
	2000	1946	88	4.5	97.3	1910	89	4.7	95.5
	10 000	9685	390	4.0	96.9	9798	311	3.2	98.0
CSF	20	20.4	0.9	4.4	102	21.2	1.7	8.0	106.0
ng/ml	100	96.8	3.9	4.0	96.8	98.6	5.1	5.2	98.6
	500	491	18.2	3.7	98.2	488	13.7	2.8	97.6
	2000	1988	55	2.8	99.4	1933	54	2.8	96.7
	5000	4860	112	2.3	97.2	4894	102	2.1	97.9
Brain	25	23.1	2.5	10.8	92.4	21.9	1.8	8.2	87.6
ng/g	100	92.7	5.6	6.0	92.7	94.1	6.3	6.7	94.1
	500	459	28.1	6.1	91.8	465	31	6.7	93.0
	2000	1894	132	7.0	94.7	1921	121	6.3	96.1
	5000	4887	270	5.5	97.7	4698	265	5.6	94.0

Table 4
Recovery values obtained from repeated analysis of plasma and brain tissue samples compared with pure standard ($n=3$)

Compound	Plasma		Brain	
	added amount (ng/ml)	Recovery % (Mean±SD)	Added amount (ng/g)	Recovery % (Mean±SD)
N	100	97.9±3.0	100	91.3±8.3
	2000	98.3±2.8	2000	94.5±5.2
	8000	97.5±2.8	5000	92.1±3.9
M1	100	93.4±5.8	100	96.0±5.1
	2000	95.3±3.3	2000	95.3±4.3
	8000	96.1±3.4	5000	94.5±6.3
IS	100	95.3±6.1	100	92.6±6.8
	2000	97.4±2.4	2000	93.8±4.6
	8000	96.8±2.7	8000	94.2±3.9

Table 5
Stability study. Concentrations are given as ng/ml for plasma and CSF and ng/g for brain tissue

Sample	Low concentration: 50				High concentration: 5000			
	Nimesulide Mean±SD	C.V.	M1 Mean±SD	C.V.	Nimesulide Mean±SD	C.V.	M1 Mean±SD	C.V.
(A) Short-term stability (n=3):								
Plasma	52.1±3.5	6.7	51.4±3.8	7.4	5110±134	2.6	5186±150	2.9
CSF	50.5±2.0	4.2	52.3±1.4	2.7	4883±101	2.1	4911±145	3.0
Brain	48.7±3.3	6.8	47.6±2.8	5.9	4546±97	2.1	4723±115	2.4
(B) Freeze–thaw cycle (n=3):								
Plasma	49.4±2.8	5.7	53.0±2.4	4.5	5421±42	1.5	5120±133	2.6
CSF	52.1±1.4	2.7	51.3±1.2	2.4	5020±72	1.4	4933±76	1.5
Brain	47.1±3.1	6.6	48.3±3.1	6.4	4873±141	2.9	4841±125	2.6
(C) Long-term stability (n=3):								
Plasma	46.8±2.2	4.7	47.7±2.6	5.5	4903±111	2.3	4745±128	2.7
CSF	48.9±1.6	3.3	47.2±0.9	1.9	4937±64	1.3	5070±89	1.8
Brain	44.7±2.9	6.5	45.4±3.2	7.0	4828±130	2.7	4869±96	2.0
(D) Extract stability (n=6):								
Plasma	51.8±2.9	5.6	49.6±2.4	4.8	4897±98	2.0	4917±137	2.8
Brain	48.7±3.2	6.6	47.1±3.3	7.0	4776±104	2.2	4861±154	3.2

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