ELSEVIER

Journal of Chromatography B, 723 (1999) 293–299

JOURNAL OF CHROMATOGRAPHY B

Short communication

Improved high-performance liquid chromatographic assay for nimesulide

David J. Jaworowicz Jr., Melissa T. Filipowski, Kathleen M.K. Boje*

Department of Pharmaceutics, School of Pharmacy, University at Buffalo, Buffalo NY 14260, USA

Received 14 July 1998; received in revised form 1 December 1998; accepted 1 December 1998

Abstract

An improved, validated HPLC assay was developed for the non-steroidal anti-inflammatory agent, nimesulide. In contrast to previous methods, the present assay requires smaller plasma volumes (0.2 ml) and utilizes a commercially available, structurally similar analogue of nimesulide, NS-398. The method involves a liquid–liquid extraction procedure that can be completed within 4 h, followed by reversed-phase HPLC analysis. Briefly, the extraction protocol required toluene extraction of acidified plasma samples, followed by back-extraction of the retained toluene phase with aqueous base. The retained aqueous alkaline phase was concentrated by toluene re-extraction. The retained toluene phase was evaporated to dryness and reconstituted with 100 μl of mobile phase. Extracted samples were injected (50 μl) onto a Shandon Hypersil BDS C_{18} column (5 μm particle size; 250×4.6 mm) equilibrated with 1.0 ml/min of 68:32 (v/v) methanol–citrate (0.08 *M*)–phosphate (0.04 *M*) buffer (pH 3.0) at room temperature, with detection at 240 nm. The chromatographic run time was 12 min with retention times of 5.9 min and 9.1 min for nimesulide and NS-398, respectively. The analytical method was successfully utilized for a pilot pharmacokinetic study.

Keywords: Nimesulide

1. Introduction

Nimesulide (Fig. 1) {methanesulfonamide, *N*-[4-nitro-2-phenoxyphenyl]-} is a selective cyclooxygenase-2 (COX-2) inhibitor with promising use for the therapeutic management of various inflammatory conditions [1]. It is currently available in several European countries. The COX-2 specificity of this nonsteroidal anti-inflammatory drug (NSAID) offers an improved analgesic and anti-inflammatory therapeutic profile without the deleterious, adverse gastrointestinal and renal effects associated with COX-1 inhibition by nonspecific NSAIDS (such as ibuprofen or indomethacin).

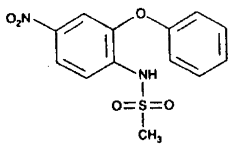
Our laboratory is interested in the potential therapeutic use of selective COX-2 inhibitors for neuroinflammatory conditions. For example, increased prostaglandin synthesis was observed in other neuroinflammatory conditions, such as meningitis, multiple sclerosis and human immunodeficiency virus-associated dementia [2–5]. Moreover, epidemiological evidence suggest that NSAIDS may beneficially retard the progression of Alzheimer's disease [6,7].

The goal of this study was to develop an analytical method for nimesulide that would be applicable to the study of pharmacokinetic–pharmacodynamic relationships of nimesulide inhibition of neuroinflam-

*Corresponding author. Tel: +1-716-645-2842, ext. 241; fax: +1-716-645-3693; e-mail: boje@acsu.buffalo.edu

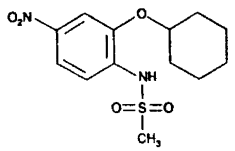
0378-4347/99/$ – see front matter
PII: S0378-4347(98)00559-3

Nimesulide



Methanesulfonamide, N-(4-nitro-2-phenoxyphenyl)-

NS-398



Methanesulfonamide, N-[2-(cyclohexyloxy)-4-nitrophenyl]-

Fig. 1. Chemical structures of nimesulide and the internal standard, NS-398.

mation. Previous analytical methods for nimesulide required large human plasma sample volumes and utilized less than ideal internal standards [8,9]. The present study presents an improved, validated analytical assay that requires smaller sample volumes and utilizes a commercially available, structurally related analogue of nimesulide, NS-398 (Fig. 1) {methanesulfonamide, *N*-[2-(cyclohexyloxy)-4-nitrophenyl]-}.

2. Experimental

2.1. Chemical

All solvents were analytical or HPLC grade, obtained from VWR (Pittsburgh, PA, USA). Nimesulide and the internal standard, NS-398 were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Male Sprague-Dawley rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN, USA). Blank rat plasma was obtained by aortic exsanguination under halothane anesthesia. All other reagents were obtained from Sigma (St. Louis, MO, USA).

2.2. Equipment

The HPLC equipment consisted of a Waters 510 pump, Waters 717+ autosampler-injector, Waters 486 variable UV–Vis absorbance detector and Hewlett-Packard HP3396 II integrator/recorder. The chromatographic column was a Shandon Hypersil BDS C_{18} column (5 μm particle size; 250×4.6 mm) with a BDS C_{18} guard column, purchased from Alltech Assoc. (Deerfield, IL, USA).

2.3. Sample preparation

2.3.1. Overview of plasma extraction procedure

The extraction procedure involves multiple liquid–liquid extractions to assure quantitative, analytical recovery of the analyte and internal standard, and to remove contaminating chromatographic interferences. The general extraction protocol involves toluene extraction of acidified plasma samples, followed by a back-extraction of the retained toluene phase with aqueous base. The analyte and internal standard are concentrated by toluene re-extraction of the retained, acidified aqueous phase; the retained toluene phase is evaporated to dryness and the residue is reconstituted for assay.

2.3.2. Sample extraction details

The detailed extraction protocol is as follows: rat plasma samples were acidified with 25 μl H_3PO_4 (1.0 *M*), vortexed and extracted for 15 min with 300 μl toluene, then centrifuged for 5 min at 10 000 *g*. The organic phase was saved and transferred to a clean tube, and the extraction process was repeated twice more with the retained acidified plasma layer. The toluene fractions from the three extractions were pooled and back-extracted for 15 min with 450 μl NaOH (0.5 *M*). Following centrifugation (5 min at 10 000 *g*), the alkaline aqueous phase was transferred to a clean tube and the retained toluene phase was back-extracted for a second time. The aqueous alkaline phases from the two extractions were pooled, acidified with 70 μl of H_3PO_4 (4.0 *M*) and re-extracted twice with 800 μl aliquots of toluene. The pooled toluene phases were retained and evaporated to dryness in a Speedvac Integrated vacuum centrifuge. Sample residues were reconstituted in

100 μl of mobile phase for subsequent HPLC analysis.

2.4. HPLC assay conditions

Extracted plasma samples (50 μl) were injected onto a Shandon Hypersil BDS C_{18} column (5 μm particle size; 250×4.5 mm) equilibrated with 1.0 ml/min of 68:32 (v/v) methanol–citrate–phosphate buffer (0.08 *M* citrate–0.04 *M* phosphate, pH 3.0) at room temperature. The citrate–phosphate buffer was prepared by mixing 80.3 ml of 0.1 *M* citric acid with 19.7 ml of 0.2 *M* dibasic sodium phosphate. Detection was at 240 nm, which is the λ_{max} for nimesulide.

2.5. Assay validation

A number of experiments were performed to validate the assay as described in published form [10–13]. The assay validation criteria were performed by two people (DJJ and MTF) using the same HPLC system.

2.5.1. Absolute recovery

Absolute recovery of nimesulide and NS-398 was determined as part of the assay validation process. In brief, blank rat plasma samples were spiked with known amounts of nimesulide and NS-398, and subjected to the previously described extraction procedure. Processed samples were assayed by HPLC, and the individual peak areas for nimesulide and NS-398 were compared to a standard curve generated from injections of analytically prepared solutions of authentic drug and internal standard dissolved in mobile phase. Standard curves were statistically analyzed by least squares linear regression (with 1/x weighting) of the peak area of authentic analyte versus theoretical mass injected, as described below.

2.5.2. Inter- and intra-day accuracy and precision

Interday and intra-day accuracy and precision were determined by spiking 1 ml of blank rat plasma with drug solution (50 μl in 70% methanol), and subdividing the spiked plasma into five aliquots (200 μl each). For the intra-day precision criteria, all samples were extracted on the same day and assayed randomly throughout the same analysis day. For the inter-day precision assessment, each of the five samples was extracted and assayed on five different days. The processed samples were quantitated as described for the absolute recovery procedures.

2.5.3. Linearity

Two different approaches were utilized in the generation of standard curves. For determination of absolute recovery, standard curves were statistically analyzed by least squares linear regression (with 1/x weighting) of the integrated peak area versus mass injected of the respective drug or internal standard. For the pharmacokinetic application, calibration curves were statistically analyzed by least squares linear regression (with 1/x weighting) of the peak ratio (nimesulide to NS-393) versus nimesulide concentration. Calibration curve linearity was assessed by analysis of variance utilizing a F test and by residual plots [14,15]. Comparison of the computed F ratio to the critical value of $F_{\alpha=0.05}$ (with the appropriate degrees of freedom) permitted statistical assessment of linearity. Calibration curves were considered linear when the statistical significance was $p<0.05$. Residual plots were assessed for systematic deviations of positive (or negative) points above (or below) zero.

2.5.4. Selectivity

To check for the possible interfering metabolites, plasma samples were obtained from a rat prior to and 75 min after intravenous dosing with nimesulide. Samples were assayed under standard analytical conditions (68:32 v/v methanol–citrate–phosphate buffer, pH 3.0) and using different mobile phases of: (a) different methanol organic strengths (25:75, 45:55, 55:45, 65:35 v/v methanol–citrate–phosphate buffer, pH 3.0); (b) of different buffer pHs (68:32 v/v methanol–citrate–phosphate buffer, pH 5.0; 68:32 v/v methanol–citrate–phosphate buffer, pH 7.0); (c) and of different acetonitrile organic strengths (40:60; 50:50; 60: 40; 80:20 v/v acetonitrile–citrate–phosphate buffer, pH 3.0).

2.6. Pharmacokinetic application

All animal studies were approved by the University's Institutional Animal Care and Use Committee,

and were performed following the NIH guidelines for proper care and use of laboratory animals. One day before drug dosing, rats were surgically cannulated at the jugular vein as previously described [16,17]. On the day of the study, nimesulide in 70% ethanol–saline was intravenously dosed as a 1 mg/kg bolus. Heparinized blood samples (0.4 ml) were collected over a 6 h interval and plasma was harvested following centrifugation of the blood samples (10 000 *g* for 5 min). Samples were stored at −70°C prior to analysis. Samples were stable for at least four weeks under these storage conditions (data not shown).

Unknown rat plasma samples (200 μl) were spiked with the internal standard, NS-398 (10 μl dissolved in 70% methanol) and extracted as described. For generation of the calibration curve, aliquots of blank rat plasma (200 μl) were spiked with drug solutions (10 μl) of nimesulide & NS-398 (dissolved in 70% methanol). These calibration standards were spiked with 0.1–2 μg nimesulide+ 2.0 μg NS-398 added to 0.2 ml of blank rat plasma. The concentrations of nimesulide spiked into the spiked calibration standards were 0.5, 1.0, 2.0, 5.0, 10.0, 12.0 and 15.0 μg/ml, while the concentration of spiked NS-398 was constant (10 μg/ml) for all calibration standards. All samples (unknown and calibration) were extracted concurrently. Calibration standards were assayed randomly throughout the analysis day. Calibration curves were generated by least squares linear regression (1/x weighting) of the peak area ratio (nimesulide to NS-393) versus nimesulide spiked concentration. For the unknown samples, the peak area ratios (nimesulide to NS-398) were calculated from the integrated peaks of the chromatograms, and then converted to nimesulide concentrations using the linear regression parameters (slope and intercept). The subsequent nimesulide concentration-time data were analyzed by noncompartmental pharmacokinetic methods [18].

3. Results and discussion

Several areas are important for assay validation: linearity, recovery, accuracy, precision and specificity [10–13]. The chromatographic retention times for nimesulide and NS-398 were typically 5.9 and 9.1 min, respectively (Fig. 2) as assayed from spiked rat plasma. Assay of plasma obtained from drug-naive rats showed that there were no endogenous interfering peaks eluting with retention times similar

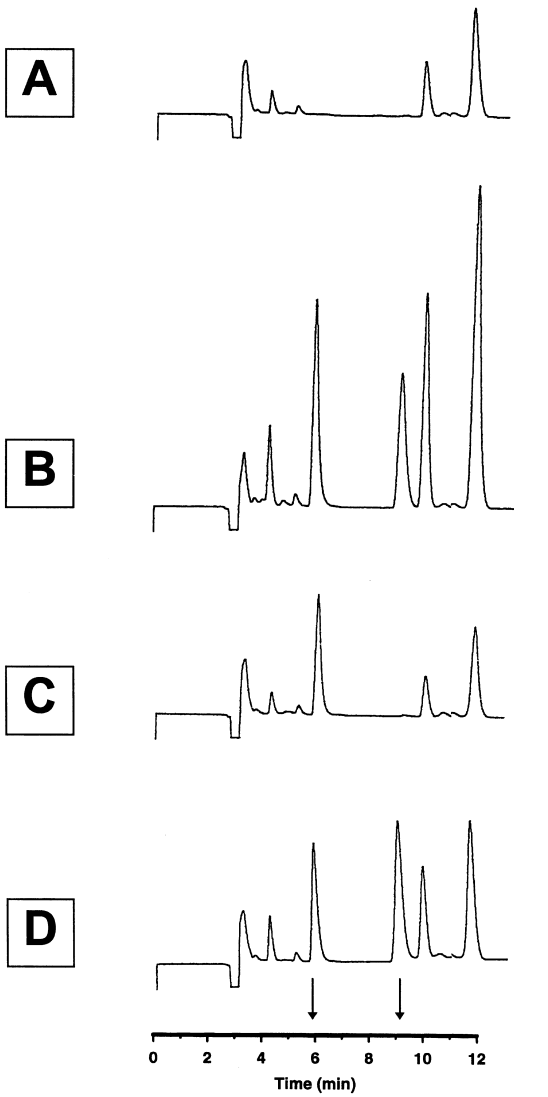


Fig. 2. HPLC chromatograms of extracted rat plasma samples. (A) Blank rat plasma. (B) Blank rat plasma spiked with nimesulide (leftmost arrow, retention time 5.9 min) and NS-398 (rightmost arrow, retention time 9.1 min). (C) Rat plasma obtained 74 min after i.v. dosing with nimesulide (1 mg/kg), without NS-398. (D) Same rat plasma, but spiked with NS-398.

to nimesulide or NS-398 (Fig. 2A). The lowest limit of detection for nimesulide was 40 ng extracted from 0.2 ml plasma (0.2 μg/ml), at a signal-to-noise ratio of 2.

3.1. Linearity

The linearity of nimesulide calibration curves was independently determined from: (a) authentic solutions for absolute recovery studies; and (b) spiked plasma samples for the pharmacokinetic application. In the determination of absolute recovery, the calibration curves of authentic nimesulide peak area versus mass injected were linear over the concentration range of 0.5–10 μg/ml, as determined by statistically significant F-ratios. Furthermore, residual analysis plots revealed no systematic bias. The spiked plasma calibration curves (based on the peak area ratio of nimesulide/NS-398 versus nimesulide concentration) were also linear, as evidenced by significant F-ratios, lack of systematic bias via residual plot analysis, and typical correlation coefficients of 0.999. The inter-day variability in the slopes and intercepts of the plasma spiked nimesulide calibration curves ($n=5$) were 0.113 ± 0.008 (RSD=7.1%) and 0.017 ± 0.012 (RSD=71.7%), respectively.

3.2. Recovery: inter- and intra-day accuracy and precision

The inter-day absolute recovery of nimesulide from spiked plasma averaged 94.8% over the concentration range of 0.5–10 μg/ml, as determined by a ratio of slopes of the calibration curves (e.g., spiked plasma calibration curve/authentic nimesulide calibration curve). The absolute recovery of nimesulide ranged from 91.5 to 96.2% (Table 1), as calculated from the peak area of the extracted plasma sample and the calibration curve of peak area versus mass injected of authentic nimesulide. NS-398 inter-day absolute recovery was ~97% for 2.5–10 μg/ml NS-398 (Table 1). The mean relative error (a measure for accuracy) over the concentration range of 0.5–10 μg/ml averaged −5.8% for nimesulide and −3.2% for NS-398. The inter-day relative standard deviation (a measure of precision) was <7% for both nimesulide and NS-398. The intra-day relative standard deviation was also <7% for nimesulide.

3.3. Selectivity

Plasma samples from a rat dosed with nimesulide were assayed for the possibility of interfering metabolites. Assay of the pre-dose plasma sample showed that endogenous peaks did not interfere with

Table 1
Assay validation data: absolute recovery, accuracy and precision

Theoretical spiked conc. (μg/ml)	Experimentally detected conc. (μg/ml)[a]	Absolute recovery from plasma (%)	Inter-day accuracy (% mean relative error)[b]	Inter-day precision (RSD %)	Intra-day precision (RSD %)
Nimesulide					
10.0	9.42±0.62	94.2±6.2	−5.8	6.6	4.2
5.00	4.81±0.31	96.2±6.2	−3.8	6.5	2.6
2.50	2.29±0.12	91.5±4.8	−8.4	5.3	nd
1.00	0.95±0.04	95.3±4.2	−5.0	4.4	6.6
0.50	0.47±0.02	94.9±3.4	−6.0	3.6	7.2
NS-398					
10.0	8.96±1.1	89.6±1.1	−10.4	1.2	nd
5.00	5.10±0.20	101.9±4.0	+2.0	4.0	nd
2.50	2.47±0.17	99.2±6.9	−1.2	7.0	nd

[a] Each analyte concentration was determined based on $n=5$ samples. Data are expressed as mean±SD.
[b] % Mean relative error=(mean experimental detected conc.−theoretical spiked conc.)/theoretical spiked conc.*100%.
nd=not done.

nimesulide or NS-398 (data not shown). Assay of rat plasma samples without (Fig. 2C) and with spiked NS-398 (Fig. 2D) revealed a nimesulide peak at 5.9 min (Fig. 2C and D) and a peak at 9.1 min, corresponding to NS-398 (Fig. 2D). No additional peaks were observed, suggesting that no other metabolites were detected by the method.

Hydroxylated nimesulide, a pharmacologically inactive metabolite formed by cytochrome P450 oxidative metabolism [8], was not detected by our analytical procedure. To assure that this major metabolite was not exactly co-eluting with nimesulide under our chromatographic conditions, we injected extracted plasma samples from a rat dosed with nimesulide using various mobile phases. The expectation was that the more polar hydroxylated nimesulide metabolite, if present in the extracted sample, might elute earlier than nimesulide under lower organic solvent strength conditions. However, no additional peaks were observed in the chromatograms following elution with the different mobile phase compositions (data not shown). While the selectivity of the analysis method is not proven conclusively by this approach, the probability of method non-selectivity is reduced by these chromatographic observations involving alterations in mobile phase composition.

3.4. Pharmacokinetic application

Fig. 3 shows individual nimesulide plasma concentration-time course data following intravenous dosing of nimesulide to two individual rats. Based on a noncompartmental pharmacokinetic analysis approach, nimesulide clearance was ~0.51 ml/min/kg; the volume of distribution was ~208 ml/kg; the mean residence time was ~552 min and the terminal half-life was ~386 min (~6.44 h).

In summary, the goal of this study was to develop an improved analytical assay for nimesulide. Compared to previously published analytical methods [8,9], the present study utilizes a better internal standard, NS-398, which is structurally similar to nimesulide and is readily available from commercial sources. Because of the structural similarities, NS-398 is expected to possess similar physicochemical properties similar to nimesulide, which is important for comparable quantitative extraction from plasma

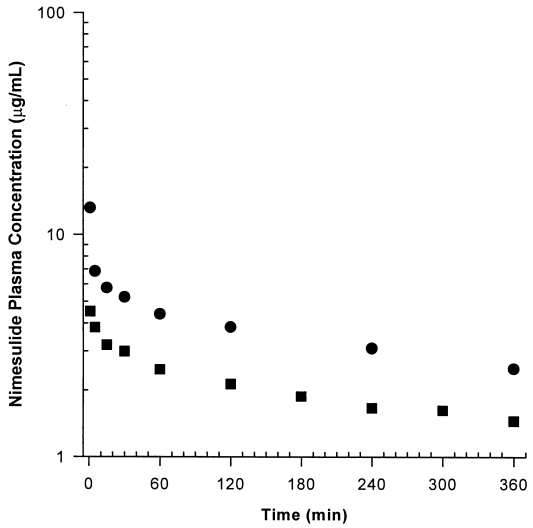


Fig. 3. Nimesulide concentration versus time in two individual rats following 1 mg/kg intravenous bolus dosing. The different symbols correspond to individual rats.

and subsequent chromatographic resolution. Previous papers utilized the structurally dissimilar tolbutamide [8] or a custom synthesized nimesulide analogue [9]. The present study also demonstrated comparable recoveries from substantially smaller sample volumes (0.2 ml versus 1–2 ml plasma). The extraction process can be performed within 4 h, and the short analytical run times permit a throughput of ~6 samples/h. Lastly, the present method provides analytical validation, consistent with the guidelines recently promulgated by Linder and Wainer [13].

Acknowledgements

Supported in part by NIH NS31939.

References

[1] J.P. Famaey, Inflammation Res. 46 (1997) 437.
[2] A.W. Johnson, Journal of Neurology, Neurosurgery & Psychiatry 58 (1995).
[3] M.M. Mustafa et al., Am. J. Dis. Child. 144 (1990) 883.
[4] D.E. Griffin, S.L. Wesselingh, J.C. McArthur, Ann. Neurol. 35 (1994) 592.
[5] D.J.J. Jaworowicz, P.J. Korytko, S.S. Lakhman, K.M.K. Boje, Brain Research Bull 46 (1998) 541.

[6] C.M. Beard, S.C. Waring, P.C. O'Brien, L.T. Kurland, E. Kokmen, Mayo Clin Proc 73 (1998) 951.
[7] G.M. Pasinetti, J. Neurosci. Res. 54 (1998) 1.
[8] D. Castoldi, V. Monzani, O. Tofanetti, J. Chromatogr. 425 (1988) 413.
[9] S.F. Chang, A.M. Miller, R.E. Ober, J. Pharm. Sci. 66 (1977) 1700.
[10] H.T. Karnes, G. Shiu, V.P. Shah, Pharmaceutical Res. 8 (1991) 421.
[11] E. Debesis, J.P. Boehlert, T.E. Givand, J.C. Sheridan, Pharmaceutical Technology, Sept. (1982) 121.
[12] A.J. Vanderwielen, E.A. Hardwidge, Pharmaceutical Technology, Mar. (1982) 66.
[13] W. Linder, I.W. Wainer, J. Chromatography B 707 (1998) 1.
[14] J.H. Zar, Linear regression, Biostatistical Analysis, Prentice-Hall, Inc, Englewood Cliffs, NJ, 1984, Ch. 17.
[15] S. Bolton, Pharmaceutical statistics: practical and clinical applications, in: J. Swarbrick (Ed.), 2nd ed, Drugs and the Pharmaceutical Sciences, Vol. 44, Marcel Dekker, New York, 1990, p. 646.
[16] K.M.K. Boje, Brain Res. 720 (1996) 75.
[17] K.M.K. Boje, J. Pharmacol. Exp.Ther. 274 (1995) 1199.
[18] M. Gibaldi, D. Perrier, Pharmacokinetics, Marcel Dekker, New York, 1982.