The Biotransformation and Pharmacokinetics of 14C-Nimesulide in Humans Following a Single Dose Oral Administration

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Abstract

Nimesulide is a preferential cyclo-oxygenase-2 inhibitory non-steroidal anti-inflammatory drug, which is licensed in over 50 countries worldwide for use as an anti-inflammatory, antipyretic and analgesic agent [1]. The pharmacological and clinical features of the agent have been reviewed [1-4] and details about the drug reported in extenso in a monograph [1]. The pharmacokinetics of nimesulide in man have been previously investigated and these studies have been reviewed by Bernareggi [5], Bernareggi and Rainsford [6] and Bjarnason et al. [7]. Nimesulide is a neutral drug (pKa 6.4; solubility in water 5.5-11.4; log P

The mean elimination half-life of total radioactivity in the plasma and whole blood was circa 4.8 h; the ratio whole blood and plasma being circa 0.6 h. The mean elimination half-lives for nimesulide and 4-hydroxynimesulide in plasma were circa 2.5 h and circa 3.9 h, respectively. The drug was rapidly excreted and recoveries were 59-66% in the urine and 33-39% in the faeces at 168 hours.

A total of 16 metabolites were identified including the conjugated metabolites, which exceeds the 5 previously identified. Nimesulide was to be metabolised by 5 pathways involving (a) cleavage of the molecule at the ether linkage (b) reduction of the NO2 group to NH2, and (c) ring hydroxylation followed by conjugation with either glucuronic acid or sulphate. In conclusion, the biotransformation pathway for nimesulide in man has now been comprehensively determined with 92% of the urinary metabolites fully characterised. The identification of some rare metabolites of nimesulide may help in understanding the mechanisms of hepatotoxicity from this drug.

Keywords: Nimesulide; Anti-inflammatory drug; Pharmacokinetics; Mass spectrometric analysis

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may give insight into the potential role of these in the aetiology of hepatic reactions associated with this drug [7,20-24]. A brief summary of the results of this study has been included in a review of the pharmacokinetics of nimesulide [7].

Methods

To enable characterisation of the biodisposition of nimesulide, the plasma kinetics and the rates and routes of excretion of total radioactivity were investigated following a single oral administration of \(^{14}\text{C}\)-nimesulide to 4 healthy male volunteers at a dose of 100 mg per subject. In addition, nimesulide and its principal metabolite, 4-hydroxynimesulide, were measured in plasma to allow the comparison of their concentrations with total radioactivity. The metabolites present in plasma, urine and faeces were investigated using chromatographic techniques. These samples were first extracted and partially purified prior to analysis by LC-MS/MS.

The male volunteers 30 to 55 years of age and had no clinically important abnormal physical or laboratory findings at the pre-screening examination and underwent screening within 14 days of commencement of the study. They had no history of alcohol or drug abuse (confirmed by Toxilab™ screen), positive test results of tests of HBs-Ag, HCV or HIV Ab, presence of drug allergy or existence of any surgical or medical conditions that might interfere with the pharmacokinetics of the test drug.

On the evening prior the day of dosing they were admitted to the clinic and fasted from 2300 h until approximately 4 h after dosing the next day. Approximately 200 ml of tap water was administered 30 min prior to dosing. Standardised meals were served at regular intervals during the study. Water, fruit juice (excluding grapefruit juice) and decaffeinated drinks were allowed from approximately 4 h post dose. Alcohol was not allowed from 48 h prior to dosing until the end of the study. The study was conducted in accordance with the provisions of the Declaration of Helsinki, 1964, with subsequent amendments approved by a local ethics committee and the exposure of volunteers to radioactive drug approval by the UK Department of Health Administration of Radioactive Substances Advisory Committee (ARSAC).

Chemicals

\(^{14}\text{C}\)-Nimesulide (4-nitro-2-phenoxymethanesulphon [U.\(^{14}\text{C}\)anilide]) was supplied by Amersham International plc (Buckinghamshire, UK) with a radiochemical purity of 99% and a specific activity of 740 MBq/mmol. The purity of the radiolabelled material was confirmed by TLC as being >97% immediately prior to administration of the dose of labelled drug. The TLC was conducted on glass backed Silica gel60 F254 of 2 mm thickness (Merck, Darmstadt, Germany) using a mobile phase of chloroform:methanol (trichloroacetimidoyl)-α-D-glucopyranuoate (450 mg, 0.82 mmol) was added, followed by 3 drops of boron trifluoride etherate. The mixture was again stirred overnight. The solvent was removed and the residue was chromatographed on silica gel with dichloromethane-methanol-acetic acid (75:20:5) to give the glucuronide triacetate of M6-G (310 mg, 82%). This was chromatographed on silica gel with dichloromethane-methanol-acetic acid (98:2) and the methyl ester triacetate of M6-G (310 mg, 82%) was supplied by Helsinn Healthcare (Lugano, Switzerland). M6 (2’-hydroxy-4’-nitro-methanesulphonanilide) and their respective phenolic glucuronides M6-G and M7-G were synthesized as described below.

Quickszint-1™ liquid scintillation fluid was obtained from Zinsser Analytic, Maidenhead, UK. Carbo-Sorb® CO\(_2\) absorbing fluid and Permafluor® scintillation fluid were used in conjunction with the Packard Tri-Carb 306 automatic sample oxidiser and were supplied by Canberra Packard Limited, Pangbourne, UK. Spec-Chec®-[\(^{14}\text{C}\)] used to estimate efficiencies of combustion, was supplied by Canberra Packard Limited, Pangbourne, UK. Acetonitrile and methanol were of HPLC grade and were supplied by Rathburn Chemical Company, UK. Orthoboric acid (AnalaR grade), chloroform (‘HiPerSol’ grade) and triethylamine (AnalaR grade) were supplied by BDH chemical company, Lutterworth, UK. Potassium hydroxide pellets (‘AR’ grade) and potassium dihydrogen orthophosphate (‘AR’ grade) were supplied by Fisons Chemical Company, Loughborough, UK.

Synthesis of M6

2-Amino-5-nitrophenol (Aldrich) (7.7 g, 50 mmol) was dissolved in pyridine (50 ml), methanesulphonyl chloride (12.6 g, 110 mmol) was added and the mixture was refluxed for 3 h. The mix was then cooled and poured into excess 5 M HCl. The solid product (the N,O-bis sulphonamide) was filtered off, washed with water and then refluxed with 2 M NaOH (150 ml). The reaction was followed by TLC until complete hydrolysis of bis-sulphonamide had occurred (ethyl acetate-petrol, 1:1, R\(_f\) of bis-sulphonamide 0.7, R\(_f\) of M6 0.5). The solution was then acidified with HCl and the crude M6 was filtered off, washed with water, dried and recrystallised from ethyl acetate-petrol. (Yield 5.4 g, 47%). \(\delta\)\_1H (250 MHz; d\_6-DMSO): 3.13 (3H, s, CH\(_3\)), 7.49 (1H, d, J 8.9, ArH), 7.67 (1H, d, J 2.6, ArH), 7.73 (1H, dd, J 8.9, 2.6, ArH), 10.20 (2H, br s, NH+OH).

Synthesis of M7

Nimesulide (1 g), iron powder (2 g) and ammonium chloride (1 g) were added to ethanol (40 ml) and water (20 ml) and the mixture was refluxed for 3 h, cooled and filtered. The filtrate was evaporated to give the crude amine (0.8 g) which was dissolved in 2 M HCl (10 ml) and cooled to 0°C before slowly adding sodium nitrite (0.2 g) dissolved in water (5 ml). The mixture was stirred for 15 mins at ~ 0°C and then added slowly to boiling water (50 ml). After 5 mins, the mixture was cooled and extracted with ethyl acetate. Evaporation of the ethyl acetate gave the crude product which was then chromatographed on silica gel with ethyl acetate-petroleum ether (3:7) to give the product M7. (Yield 0.45 g, 50%). \(\delta\)\_1H (250 MHz; d\_6-DMSO): 2.90 (3H, s, CH\(_3\)), 6.19 (1H, d, J 2.3, ArH), 6.49 (1H, dd, J 8.5, 2.4, ArH), 7.13 (4H, m, ArH), 7.42 (2H, m, ArH), 9.00 (1H, s, NH+OH), 9.62 (1H, s, NH+OH).

Synthesis of M6-G

The phenol M6 (160 mg, 0.69 mmol) was suspended in dry dichloromethane (10 ml) and methyl 2,3,4-tri-O-acetyl-1-O- (trichloroacetimidoyl)-α-D-glucopyranoside (450 mg, 0.82 mmol) was added, followed by 3 drops of boron trifluoride etherate. The mixture was stirred overnight but TLC (ethyl acetate petroleum, 1:1) showed only partial reaction had occurred. A further portion of imidate (200 mg) and 3 drops of boron trifluoride etherate were added and the mixture was again stirred overnight. The solvent was removed and the residue was chromatographed on silica gel with dichloromethane-methanol (98:2) to give the methyl ester triacetate of M6-G (310 mg, 82%). This was added to 1 M NaOH (6 ml) and the mixture was left overnight, acidified with 1 M HCl and then extracted with ethyl acetate. The ethyl acetate was evaporated and the residue was chromatographed with dichloromethane-methanol-acetic acid (75:20:5) to give the glucuronide M6-G as an off white solid. (Yield 120 mg, 52%); mass spectrum (FAB) 409 (M‘+H), 232 (M‘-176); \(\delta\)\_1H (250 MHz; CD_OD) 3.10 (3H, s, CH\(_3\)), 7.45 (4H, m, ArH), 9.00 (1H, s, NH+OH), 9.62 (1H, s, NH+OH).
Synthesis of M7-G

Using exactly the same method as described above for the preparation of M6-G, the phenol M7 was converted to the glucuronide M7-G, an off white solid. (Yield 43%); mass spectrum (FAB) 456 (M+H), 279 (M–176); δ(300 MHz, CDCl3) 2.39 (3H, s, CH3), 3.30–3.52 (3H, m, 3×CHOH), 3.84 (1H, d, J 9.4, OCH3CO), 4.84 (1H, m, OCHO), 6.55 (1H, d, J 2.6, ArH), 6.82 (1H, dd, J 8.8, 2.6, ArH), 7.15 (3H, m, ArH), 7.40 (3H, m, ArH).

Dose preparation and administration

The specific activity of [14C]-nimesulide supplied by Amersham (740 MBq/mmol) was confirmed during dose formulation and the specific activity of free base [14C]-nimesulide thus calculated to be 0.021 MBq/mg was the total amount of [14C]-nimesulide in the final formulation.

The [14C]-nimesulide was prepared for the dose administration by combining 7.03 mg (16.6 MBq) of [14C]-nimesulide and 795 mg of non-radiolabelled nimesulide in ethyl acetate. The ethyl acetate was removed under Ne. The test material was placed in a teflon shaking flask containing grinding balls and placed on a Microdismembrator-II™ (Braun Medical, Melsungen, Germany) to break down the particles so that less than 5% of the particles counted exceeded 5 μm in diameter. The particle size of the nimesulide was using an Aerosizer (Amherst Process Instruments, Tewkesbury, England) and light microscopy.

The target dose was 100 mg and this was formulated as a dry powder in gelatin capsules. The specific activity of the dose was such that each subject received a target radioactive dose of 2.1 MBq (0.5 mSV). This dose complied with the `International Commission on Radiological Protection’ (ICRP) Guidelines (1992) for a Category IIa study (0.1–1 mSV). The actual dose received by each subject was calculated using the weight and specific activity of [14C]-nimesulide in the formulated dose.

Collection of biological samples

Urine samples were collected quantitatively at 0–2, 2–4, 4–6, 6–8, 8–12, 12–24, 24–48, 48–72, 72–96, 96–120, 120–144 and 144–168 h post dose. Faeces were collected over 24 h periods after dose administration up to 168 hours. Venous blood samples (ca 12 ml) were collected from in situ venous cannula or by venepuncture into lithium heparinised tubes at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 14, 18, 24, 48, 72, 96, 120, 144 and 168 h post dose. Aliquots of blood (ca 1 ml) were immediately transferred to a lithium heparinised tube for the determination of radioactivity in whole blood. Plasma was separated from the remaining blood by centrifugation (3000 rpm for 10 min) and divided into 2 tubes. The level of total radioactivity was measured in each sample of urine, faeces, plasma and whole blood collected.

Analysis of samples for total radioactivity

Duplicate aliquots of urine and plasma were taken for the determination of radioactivity. Plasma aliquots were made up to 1 ml with water and mixed with Quickszint-™ scintillation fluid (10 ml).

Faecal samples were homogenised in water:acetonitrile (50:50) and duplicate sub-samples were weighed into Combustocoones® (Packard Instruments Company Limited) and combusted using a Packard Tri-Carb™ 306 Automatic Sample Oxidiser (Canberra Packard Limited). The resultant 14CO2 generated was collected by absorption in Carbo-sorb™ CO2 absorbing fluid (8 ml) and Permaflour E:™ scintillation fluid (10 ml) added prior to analysis by Liquid Scintillation Analysis. Duplicate aliquots of each blood sample were also combusted using a Packard Tri-Carb™ 306 Automatic Sample Oxidiser. Combustion of standards during faecal and whole blood sample combustion showed that recovery efficiencies were in excess of 97% throughout.

Solid phase extraction of urine samples

Waters 3cc Oasis® cartridges (HLB 3cc, 60 μg) were washed with 2 ml of acetonitrile following by 2 ml of 1% acetic acid in water (v/v). The urine samples were acidified with acetic acid and then applied to a prepared cartridge. The column eluate was collected, the total volume was measured and aliquots taken for LSC. The column was then washed sequentially with 2 ml of each of hexane, ethyl acetate, ether, acetonitrile and methanol. Following each solvent wash the eluate was collected, the total volume was measured and aliquots were taken for LSC to calculate the extraction efficiency and the distribution of radioactivity in the solvent fractions. Each of the fractions were taken to dryness under nitrogen and re-dissolved in acetonitrile:water (1:1, v/v) prior to LC-MS/MS.

Flash column chromatography of urine samples

The methanol fraction from the solid phase extraction procedure was further processed by flash column chromatography. A silica column was prepared in a mobile phase of chloroform:methanol:formic acid (75:25:1, by volume). The methanol fraction was dried and redissolved in the mobile phase. The sample was applied to the column and eluted sequentially with chloroform:methanol:formic acid (75:25:1, v/v/v), chloroform:methanol:formic acid (50:50:1, v/v/v), chloroform:methanol:formic acid (25:75:1, v/v/v) and methanol:formic acid (100:1, v/v). Fractions (ca 0.5 ml) were collected and aliquots taken for LSC. Fractions were pooled when they were common to peaks of radioactivity and the pooled fractions were dried under nitrogen then re-dissolved in HPLC mobile phase prior to analysis by LC-MS/MS.

Enzyme hydrolysis of urine samples

A 10 ml sample of pooled urine was dried under nitrogen and the residue re-dissolved in 10 ml of methanol. The sample was centrifuged (3000 rpm for 10 min) and the supernatant decanted. The methanol fraction was dried under nitrogen and the residue re-dissolved in 10 ml of water and aliquots taken for LSC. The overall recovery of radioactivity was 81%. A 1 ml sub-sample was retained for HPLC analysis and the remaining 9 ml was placed in a vial with 1000 units of Escherichia coli-derived β-glucuronidase (Sigma, phosphate buffered to pH 6.8). The sample was placed in a water bath at approximately 37°C for ca 16 h.

Extraction of enzyme hydrolysed urine

Enzyme hydrolysed urine was extracted using 25 ml Isolute™ C18 SPE cartridges that were washed with 5 ml of acetonitrile following by 10 ml of 1% aqueous acetic acid. The hydrolysed urine sample was mixed with 9 ml of 1% aqueous acetic acid and applied to the column. The column eluate was collected, the total volume measured and
aliquots taken for LSC. The column was then washed sequentially with 10 ml of each of 1% aqueous acetic acid in water (SPE fraction 1), 1% aqueous acetic acid in water.acetonitrile (95:5, v/v) (SPE fraction 2), 1% aqueous acetic acid in water:acetonitrile (90:10, v/v) (SPE fraction 3), 1% aqueous acetic acid in water:acetonitrile (75:25, v/v) (SPE fraction 4), 1% aqueous acetic acid in water.acetonitrile (50:50, v/v) (SPE fraction 5), and 1% aqueous acetic acid in acetonitrile (SPE fraction 6). The eluates of each fraction were collected, the total volume measured and aliquots were taken for LSC to calculate the extraction efficiency and the distribution of radioactivity in the solvent fractions.

Solvent extraction of faecal samples

Pooled faeces samples (0–48 h from each subject) were macerated in 30 ml of methanol. The sample was centrifuged (3000 rpm for 10 min) and the organic extract was collected. The post extracted solids (PES) were re-extracted similarly with a further 2×30 ml of methanol. The extracts were combined and the total volume recorded. Two aliquots were taken for liquid scintillation counting. Sub-samples were removed from the residual solids for combustion prior to LSC. The PES remaining after methanol extraction was further extracted with methanol:water (80:20, v/v), methanol:water:ammonium hydroxide (80:20:1, v/v/v), methanol:water:acetic acid (80:20:1, v/v/v), 0.1 M HCl, overnight hydrolysis at 40°C and 0.1 M HCl, overnight hydrolysis at 40°C. The total volume of each extract was recorded and aliquots taken for LSC to establish the extractable portion of the radioactivity.

Papain digestion of faecal samples

Pooled faecal samples (10 g) were each mixed with 100 ml of 20 mM sodium phosphate buffer (pH 6.8), containing 1 mM EDTA, 2 mM dithiothreitol and ca 300 µg/ml papain which was used to enable full digestion of faecal proteins. The sample was thoroughly shaken and then incubated at 60°C for ca 60 min. At the end of this time the sample was removed from the incubation oven and ca 100 ml of ice-cold methanol was added to terminate the reaction. The post-incubate was then stored in the fridge at 4°C for ca 48 h. Upon removal from the fridge the post-incubate was allowed to equilibrate to room temperature prior to being centrifuged at ca 3000 rpm for ca 10 min. The total volume of the extract was recorded and two aliquots were taken for LSC. Sub samples were removed from the final PES for oxidation prior to LSC.

Chromatographic analysis

Metabolites of nimesulide were separated on a Inertsil™ ODS 2 (25 cm×4.6 mm, 5 µm) HPLC column and an HP1050 series HPLC. Gradient elution was used, mobile phase A comprised 50 mM ammonium acetate-acetonitrile (80:20 v/v) and mobile phase B was 100% acetonitrile. Mobile phase A was used for the initial 10 minutes of each run and then a linear gradient over 30 minutes to 75% mobile phase B was used. A flow rate of 1 ml per minute was used and metabolite were detected using a Packard Radiomatic™ Flo-one® / Beta, Flow Scintillation Analyser (Model 150TR). The HPLC method used an Inertsil™ ODS 2, 10×3.2 mm i.d., 5 µm particle size guard column and an Inertsil ODS2, 250×4.6 mm i.d., 5 µm particle size analytical column. The column was maintained at 30°C and a flow rate of 1 ml/min was used. Mobile phase A was acetonitrile:25 mM KH2PO4 containing 0.1% triethylamine and pH corrected to 7.5 with 10 M KOH 4060 (v/v) and mobile phase B was acetonitrile:25 mM KH2PO4 containing 0.1% triethylamine and pH corrected to 7.5 with 10 M KOH 7030 (v/v). A solvent gradient was used to separate nimesulide and 4-hydroxynimesulide. 100% of mobile phase A was pumped for the initial 10 minutes followed by a linear gradient to 100% of mobile phase B over 2 minutes. This was maintained for a further 4 minutes. Analytes were detected using a variable wavelength uv detector set at 230 nm. The retention times of 4-hydroxynimesulide, internal standard and nimesulide were 4.5, 6.6 and 7.4 minutes, respectively. The analytical method was validated for use in human plasma over the concentration range 50 ng/ml to 50 µg/ml for each compound. The accuracy and precision at this level for nimesulide were 97.1% and 7.5%, respectively. The accuracy and precision at this level for 4-hydroxynimesulide were 100.6% and 6.6%, respectively. The extraction procedure was shown to give a quantitative recovery of nimesulide, 4-hydroxynimesulide, and the internal standard.

Results

The dose of radiolabelled drug was well tolerated in all of the volunteers and no clinical observations relating to drug administration were observed.

Plasma concentrations of total radioactivity

Plasma total radioactivity in the blood and plasma following oral administration of [14C]-nimesulide are shown in figure 1. The radioactivity in the urine and faeces as well as total recoveries of radioactivity are shown in figure 2. Plasma concentrations peaked at between 2 and 6 h post dose for each subject with the peak mean concentration of radioactivity occurring 4 h post dose (2.99 ± 1.02 µg equiv/ml). The plasma concentrations decreased slowly to 1.78 ± 0.43 µg equiv/ml at 8 h post dose and further to 0.88 ± 0.33 µg equiv/ml by 12 h post dose. Thereafter, the concentrations continued to decrease and by 48 h post dose were near to the limit of reliable measurement. The mean AUC for total radioactivity for the periods 0–24 h and 0–∞ were calculated to be 29.47 ± 3.97 µg equiv.h/ml and 30.77 ± 4.10 µg equiv.h/
ml, respectively. The mean elimination half life of total radioactivity was 4.8 ± 0.35 h.

**Plasma Concentrations of Nimesulide**

Plasma concentrations of nimesulide following oral administration of [14C]-nimesulide are presented graphically in figure 1. Plasma concentrations were highest between 1.5 and 6 h post dose for each subject, with the peak mean concentration occurring 4 h post dose (1.95 ± 0.67 µg/ml). Concentrations decreased slowly to 0.84 ± 0.43 µg/ml at 8 h post dose and further to 0.30 ± 0.21 µg/ml by 12 h post dose, and by 24 h post dose were below the limit of reliable measurement (0.05 µg/ml).

The mean AUC for nimesulide for the periods 0-24 h and 0-∞ were calculated to be 13.48 ± 2.43 µg.h/ml and 14.67 ± 3.23 µg.h/ml, respectively. The mean elimination half life of nimesulide was 2.5 ± 0.55 h.

**Plasma concentrations of 4-Hydroxynimesulide**

Plasma concentrations of 4-hydroxynimesulide following oral administration of [14C]-nimesulide are presented graphically in figure 1. Plasma concentrations were highest between 4 and 8 h post dose for each subject with the peak mean concentration occurring 4-6 h post dose (0.87 ± 0.41 µg/ml). Concentrations decreased slowly to 0.73 ± 0.10 µg/ml at 8 h post dose and further to 0.57 ± 0.12 µg/ml by 10 h post dose. Thereafter, concentrations continued to decrease and by 48 h post dose were below the limit of reliable measurement (0.05 µg/ml).
The mean AUC for 4-hydroxynimesulide for the periods 0-24 h and 0-∞ were calculated to be 9.12 ± 0.74 µg h/ml and 10.10 ± 0.86 µg h/ml, respectively. The mean elimination half life of 4-hydroxynimesulide was 3.9 ± 0.57 h.

Whole blood concentrations and whole blood/plasma ratio of total radioactivity

Whole blood concentrations of total radioactivity following oral administration of [14C]-nimesulide are shown in figure 1. Whole blood concentrations were highest between 1.5 and 6 h post dose. The mean peak of radioactivity (1.72 ± 0.55 µg equiv/g) occurred at 4 h post dose. The mean AUC for total radioactivity in whole blood for the periods 0-24 h and 0-∞ were calculated to be 17.57 and 18.39 µg equiv h/g, respectively. The mean elimination half life of total radioactivity was 4.8 h. In general, whole blood concentrations paralleled those in plasma but at a lower level. The whole blood/plasma ratio of mean total radioactivity was circa 0.6 at 4 h, ca 0.6 at 8 h, and ca 0.6 at 24 h, which suggests the radiolabelled components were not associated with blood cells.

Excretion of total radioactivity

The cumulative excretion of total radioactivity following oral administration of [14C]-nimesulide is presented graphically in figure 2. The recovery of radioactivity was quantitative in all subjects, ranging from 98.0-99.1% (mean value 98.7%). The major route of excretion was via the urine, accounting for 59-66% (mean 62.5%) of the administered dose. Radioactivity recovered in the faeces accounted for 33-39% (mean value 36.2%). The 48 h faecal sample from Subjects #1 and #2 were combined at analysis in error. The percentage dose of total radioactivity in this combined sample was 64.1%. A value of 32.0% was equally attributed to Subjects #1% and #2% and used in subsequent cumulative excretion calculations. Recovery of radioactivity in the excreta was rapid with 91-95% (mean 93.3%) of the dose recovered in the first 48 h post dose.

Solid phase extraction of urine

When a urine pool was applied to a SPE cartridge, 90.2% of the matrix radioactivity was retained. The radioactivity distributed in hexane, ethyl acetate, ether, acetonitrile and methanol fractions contained 0.0%, 12.9%, 2.1%, 19.9% and 42.6% of the urinary radioactivity, respectively, giving an overall recovery of 77.5% total recoverable radioactivity (TRR). The ethyl acetate, acetonitrile and methanol fractions were analysed by LC-MS. In addition, the methanol extract was further fractionated by flash column chromatography prior to analysis by LC-MS.

Solid phase extraction of enzyme hydrolysed urine

When an enzyme hydrolysed urine sample was applied to a SPE cartridge, 99.5% of the matrix radioactivity was retained. The radioactivity was distributed in fractions as detailed in table 1. The bulk of the radioactivity (87.5%) was eluted in the later fractions (SPE fractions 5a-7a) which contained a greater proportion of acetonitrile.

Each of these fractions was analysed by LC-MS.

Sequential solvent extraction of faeces

The methanol extract of faeces gave an extractability of approximately 53.9% TRR; the further extraction of the residual solids with methanol/water 80:20 v/v gave an additional 1.4% TRR. Altering the pH of the extraction solvent (methanol/water 80:20 v/v) with ammonium hydroxide or acetic acid gave a further 1.3% or 0.9% TRR, respectively. Overnight hydrolysis (x2) of the residual solids with 0.1 M HCl at 40°C gave a further 8.7% TRR. The total extractability from this sample was 66.2% TRR. Digestion of faecal samples with papaain resulted in an overall extractability of 57.6%.

LC-MS/MS

The molecular ions, fragment ions and daughter obtained during LC-MS/MS of metabolite standards and the respective metabolites are shown in table 2.

Reference standards

Nimesulide yielded a deprotonated molecular ion at m/z 307 and an ion at m/z 229 which corresponded to the loss of SO2CH3 from the deprotonated molecular ion. The daughter spectrum of the ion at m/z 307 was dominated by the loss of 78 (SO2CH3) to yield the ion at m/z 229. The ion at m/z 228 (loss of 79; SO2CH2) was also present but was much less intense than the corresponding ion observed for reference standards M2 (BBR 2335-02) and M3 (BBR 2395-03). This was also the case for the ion at m/z 79 which was much less intense in this spectrum.

Reference standard M1 yielded a deprotonated molecular ion at m/z

Table 1: The Extraction and Distribution of Radioactivity from Enzyme Hydrolysed Urine Using Solid Phase Extraction

<table>
<thead>
<tr>
<th>Fractions</th>
<th>% of TRR</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPE fraction 1a</td>
<td>Non retained material 0.46</td>
</tr>
<tr>
<td>SPE fraction 2a</td>
<td>1% acetic acid in water 0.54</td>
</tr>
<tr>
<td>SPE fraction 3a</td>
<td>1% acetic acid in water:acetonitrile (95:5, v/v) 0.51</td>
</tr>
<tr>
<td>SPE fraction 4a</td>
<td>1% acetic acid in water:acetonitrile (90:10, v/v) 1.37</td>
</tr>
<tr>
<td>SPE fraction 5a</td>
<td>1% acetic acid in water:acetonitrile (75:25, v/v) 15.48</td>
</tr>
<tr>
<td>SPE fraction 6a</td>
<td>1% acetic acid in water:acetonitrile (50:50, v/v) 57.68</td>
</tr>
<tr>
<td>SPE fraction 7a</td>
<td>1% acetic acid in acetonitrile 14.36</td>
</tr>
<tr>
<td>Total recovery</td>
<td>90.36</td>
</tr>
</tbody>
</table>

TRR = Total radioactive residue

Table 2: Molecular ions, fragment ions and daughter ions obtained from isolated metabolites. Radiolabelled components isolated from biological matrices.
of 323 (\([\text{M-H}]\)). The spectrum also contained the ions at \(m/z\) 265 and 245. The ion at \(m/z\) 265 was not related to M1 (4-hydroxynimesulide), however, the ion at \(m/z\) 245 corresponded to the loss of \(\text{SO}_2\text{CH}_3\) from the deprotonated molecular ion. The daughter spectrum of the ion at \(m/z\) 323 was dominated by the loss of 78 (\(\text{SO}_2\text{CH}_3\)) to yield the ion at \(m/z\) 245. The ion at \(m/z\) 243 (loss of 80; \(\text{SO}_2\), \(\text{CH}_4\)) was also present but was much less intense than the corresponding ion observed for reference standards M2 (BBR 2335-02) and M3 (BBR 2395-03).

Reference standard M2 yielded a deprotonated molecular ion at \(m/z\) of 277 (\([\text{M-H}]\)). The daughter spectrum of the ion at \(m/z\) 277 contained ions at \(m/z\) 79, 197 and 199. The ion at \(m/z\) 79 (\(\text{SO}_2\text{CH}_3\)) was the base peak of this spectrum. The loss of 78 (\(\text{SO}_2\text{CH}_3\)) and 80 (\(\text{SO}_2\), \(\text{CH}_4\)) gave the ions at \(m/z\) 199 and 197, respectively.

Reference standard M3 yielded a deprotonated molecular ion at \(m/z\) of 293 and the daughter spectrum of this ion contained ions at \(m/z\) 79, 213 and 215. The loss of 78 (\(\text{SO}_2\text{CH}_3\)) and 80 (\(\text{SO}_2\), \(\text{CH}_4\)) gave the ions at \(m/z\) 215 and 213, respectively, were significant. The ion at \(m/z\) 79 was assigned as \(\text{SO}_2\text{CH}_3\).

Reference standard M4 yielded a deprotonated molecular ion at \(m/z\) of 319 (\([\text{M-H}]\)). The spectrum also contained an ion at \(m/z\) of 239 which corresponded to the loss of \(\text{SO}_2\) and \(\text{CH}_3\) from the deprotonated molecular ion. The daughter spectrum of the ion at \(m/z\) 319 contained ions at \(m/z\) 239 and 241. These ions corresponded to the loss of 80 (\(\text{SO}_2\), \(\text{CH}_4\)) and 78 (\(\text{SO}_2\text{CH}_3\)), respectively. The ion at \(m/z\) 79 was present but was not as intense.

Reference standard M5 yielded a deprotonated molecular ion at \(m/z\) of 335 and the daughter spectrum of this ion at \(m/z\) 335 showed the loss of 80 (\(\text{SO}_2\text{CH}_3\)) to yield the ion at \(m/z\) 255 and 78 (\(\text{SO}_2\), \(\text{CH}_4\)) to give \(m/z\) 257. The ion at \(m/z\) 79 was also present.

Reference standard M6 gave a deprotonated molecular ion at \(m/z\) 231 and the fragment ion at 152 produced by the loss of \(\text{SO}_2\text{CH}_3\). The daughter spectrum obtained from the base peak at \(m/z\) 231 contains an abundant ion at \(m/z\) 122 which is produced by the loss of \(\text{NO}\) from the \(m/z\) 152 ion.

The mass spectrum of reference standard M7 contained a deprotonated molecular ion at \(m/z\) 278 and a [2M-1] ion at \(m/z\) 557. The daughter spectrum obtained from the base peak at \(m/z\) 278 contained ions at \(m/z\) 198 (loss of \(\text{SO}_2\text{CH}_3\)) and \(m/z\) 79 (\(\text{SO}_2\text{CH}_3\)).

Reference standard M6-G yielded a deprotonated molecular ion at \(m/z\) 407. A fragment ion was present at \(m/z\) 231 which corresponds to the loss of the glucuronide moiety. An ion was present at \(m/z\) 815 which corresponds to [2M-1].

Reference standard M7-G yielded a deprotonated molecular ion at \(m/z\) 454. An ion was present at \(m/z\) 909 which corresponds to [2M-1].

**Identification of metabolites (for structures see figure 4)**

Nimesulide was identified in an acetone/nitrile extract of faeces and in plasma. The molecular weight (308) was confirmed by the [M-1] ion at \(m/z\) 307, the spectrum obtained was consistent with the reference standard. The daughter ion spectrum obtained from nimesulide (\(m/z\) 307) in faecal extracts contained the ion at \(m/z\) 229 which corresponds to the loss of \(\text{SO}_2\text{CH}_3\).

M1 (4-hydroxynimesulide) was identified in urine and plasma. The MW of 324 was confirmed by the [M-1] ion at \(m/z\) 323. The ion at \(m/z\) 245 is generated by the loss of \(\text{SO}_2\text{CH}_3\) and this was consistent with the reference standard. The daughter ion spectrum produced from M1 was consistent with the daughter ion spectrum obtained from the reference standard. The principle ions were \(m/z\) 245 and 243 which are produced by the loss of \(\text{SO}_2\text{CH}_3\) and \(\text{SO}_2\text{CH}_4\), respectively. The ion (\(m/z\) 323) associated with this metabolite was observed at 2 separate retention times, indicating that isomers are formed with different positions of hydroxylation.

M3 was detected in urine, however, a full scan mass spectrum was not obtained because it co-chromatographed with M5 which was more abundant. The presence of metabolite 3 was confirmed by generating selected ion chromatograms which confirmed the \(m/z\) 293 ([M-1] ion from M3) and the \(m/z\) 335 ions ([M-1] ion from M5). The \(m/z\) 293 ion is also present as a contaminating ion in the spectrum obtained for M5.

M5 was detected in deconjugated urine. The Mwr of 336 was confirmed by the ion at \(m/z\) 335. The minor ion at \(m/z\) 293 is not related to M5 but was produced by the co-elution of M3 which has a molecular weight of 294. The daughter ion mass spectrum obtained for M5 contained a major ion at \(m/z\) 255 which corresponded to the loss of \(\text{SO}_2\text{CH}_4\) and is consistent with the reference standard.

M6 was identified as a trace metabolite in deconjugated urine. It had a molecular weight of 232 which was confirmed by the ion at \(m/z\) 231 in the mass spectrum. The loss of \(\text{SO}_2\text{CH}_3\) gave the minor ion at \(m/z\) 152 which was consistent with the reference standard. The daughter ion mass spectrum was consistent with the reference standard with major ions at \(m/z\) 152, 122 and 79. The ion at \(m/z\) 152 is produced by the loss of \(\text{SO}_2\text{CH}_4\), the ion at \(m/z\) 122 is \(\text{C}_6\text{H}_4\text{NO}_2\) from the \(m/z\) 231 ion and the ion at \(m/z\) 79 represents the \(\text{CH}_2\text{SO}_2\).

M7 was confirmed in deconjugated urine by LC-MS and although the spectrum obtained was weak, a daughter ion spectrum was obtained from the molecular ion at \(m/z\) 278. The major daughter ions were \(m/z\) 198 and 79, the 198 ion is generated by the loss of \(\text{SO}_2\text{CH}_3\). Both the spectra were consistent with the reference standard spectra.

M8 was detected in urine and was identified as a glucuronide conjugate of M7 with a Mwr of 455 which gave the molecular ion at \(m/z\) 454, consistent with the reference standard. The occurrence of this metabolite was confirmed by enzyme deconjugation experiments with \(\beta\)-glucuronidase. Following the treatment of urine with \(\beta\)-glucuronidase (sulphatase free) metabolite 7 was identified by LC-MS.

M9 was identified as a sulphate conjugate of M1. The Mwr of 404 was confirmed by the deprotonated molecular ion at \(m/z\) 403, the daughter ion spectrum obtained from the 403 contained an ion at \(m/z\) 323 which was produced by the loss of \(\text{SO}_4\).

M10 was identified in urine and was assigned as a glucuronide conjugate of M1. The Mwr of 500 was confirmed by an ion at \(m/z\) 499. The daughter ion spectrum obtained from the 499 ion contained an ion at \(m/z\) 323 which was produced by the loss of the glucuronide moiety and confirms that this molecule is a glucuronide conjugate of metabolite 1. The ion at \(m/z\) 499 was shown to be abundant at 2 main positions in the urinary chromatogram which indicates that 2 positions of hydroxylation are possible. The second position of hydroxylation gave a earlier retention time than that obtained for M10 and this component was labelled M11.

The mass spectrum for M12 was obtained only following enzyme deconjugation with \(\beta\)-glucuronidase which would indicate that this metabolite was not present in the aglycone form. The Mwr of 340 is confirmed by the deprotonated molecular ion at \(m/z\) 339. The ion at \(m/z\) 231 was consistent with structure.
M13 was identified as a sulphate conjugate of M7 with a Mwr of 359. The mass spectrum obtained contained a deprotonated molecular ion at m/z 358 and an ion at m/z 279 produced by the loss of SO3 from the molecule confirming that it is a sulphate conjugate of M7.

M14 was tentatively proposed to be a glucuronide conjugate of M5, with Mwr 512. The mass spectrum obtained was weak but contained an ion at m/z 511 which was proposed to be the deprotonated molecular ion. M5 was a significant peak in urine hydrolysed with beta-glucuronidase.

M15 was identified as the glucuronide conjugate of M12, with Mwr 516. The molecular weight was confirmed by the deprotonated molecular ion at m/z 515, the minor ion at m/z 339 was produced by the loss of the glucuronic acid moiety.

M16 was identified as a sulphate conjugate of M12 and was identified in, with a Mwr 420. The molecular weight was confirmed by the deprotonated molecular ion at m/z 419. The daughter ions produced from the m/z 419 ion confirmed the structure. The ions present (m/z 339 and 259) represent the loss of 80 and 160 which corresponds to SO3 or SO2CH4 and SO3 and SO2CH4.

M17 was identified in urine as a glucuronide conjugate of M6, with Mwr of 408. The molecular weight was confirmed by the deprotonated molecular ion at m/z 407. The loss of glucuronide produces the minor ion at m/z 231. The ions observed were identical with the reference standard.

M18 is proposed to be a sulphate conjugate of M5, with a Mwt 416 that consistent with the deprotonated molecular ion is present at m/z 415. However no fragmentation information was present so this assignment is tentative.

Metabolite quantification

The radio-HPLC profile obtained from 0-24 h urine is shown in figures 3a-3e. Peaks were assigned in the chromatogram by monitoring for each of the masses associated with metabolites identified by LC-MS of urinary fractions. 0-24 h urine was found to contain 13 peaks of radioactivity, 10 of these peaks were assigned as identified metabolites.
(M1, M8, M10, M11, M14, M15, M16, M17 and M18) and a further 2 were assigned as glucuronide conjugates (retention times 2.8 and 3.2 min) because these polar peaks were not present in a sample that had been treated with β-glucuronidase. Table 3 shows the assignment of peaks in urine and each metabolite as a percentage of the dose. Identified metabolites account for 92.4% of the urinary radioactivity and 49.4% of dose (in urine). Unidentified components represented 7.6% of the urinary radioactivity and 4.1% of the dose (in urine).

Figure 3a shows the HPLC profiles of SPE fractions 5a, 6a and 7a which account for 87.52% of the radioactivity from an enzyme hydrolysed sample of 0-24 h urine. The enzyme hydrolysis was conducted with β-glucuronidase from *E. coli* which does not contain sulphatase activity, as a result the sulphate conjugates M9, M16 and M18 are present in these fractions. Other peaks present are M1, M5, M6, M7 and M12 which are aglycones of M10, M14, M17, M8 and M15, respectively further confirming the assignments made on the

![Figure 3c](image-url)  
**Figure 3c:** HPLC chromatograms of extracts of enzyme hydrolysed urine.

![Figure 3d](image-url)  
**Figure 3d:** HPLC chromatograms of extracts of enzyme hydrolysed urine.
intact glucuronide conjugates during LC-MS analysis of urine fractions (Table 3).

The radio-HPLC profile (Figure 4) obtained from acetonitrile extract of faeces contained 2 radiolabelled components which were detectable above background. The non polar component which accounted for approximately 50% of the extractable radioactivity was assigned as nimesulide. The polar component was not identified.

The concentrations of radioactivity in plasma were too low for analysis by radio-HPLC, however LC-MS analysis of plasma confirmed the presence of nimesulide and 4-hydroxynimesulide. Nimesulide and M1 were quantified using a validated bioassay and the quantities of these components present would account for virtually the entire radioactivity present in plasma (Figure 1).

### Discussion

This study gives the most comprehensive data on the metabolic transformation and recovery from the extraction pathways of nimesulide reported to date.

The mean $C_{\text{max}}$ and $T_{\text{max}}$ values obtained for nimesulide were $2.35 \pm 0.35 \mu g/ml$ and $3.88 \pm 1.84$ hours. These values are similar to the kinetic parameters measured in patients administered 100 mg of nimesulide in tablet or suspension from where the $C_{\text{max}}$ and $T_{\text{max}}$ ranges reported have been $2.86-6.50 \mu g/ml$ and $1.22-2.75$ hours [5]. While the $C_{\text{max}}$ value obtained in the present study is lower than the published range and the $T_{\text{max}}$ is later, however the elimination half life ($2.49 \pm 0.55$ hours) and AUC$_\infty$ ($16.67 \pm 3.23 \mu g/mlh$) are consistent with the published ranges of 1.96-4.73 hours for the elimination half live and 14.65-54.09 $\mu g/mlh$.

### Table 3: The Assignment of Peaks in 0-24 h Urine.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Retention Time</th>
<th>Metabolite as % Dose</th>
<th>Metabolite Names</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.8</td>
<td>3.11</td>
<td>Unknown glucuronide conjugate</td>
</tr>
<tr>
<td>2</td>
<td>3.2</td>
<td>4.30</td>
<td>Unknown glucuronide conjugate</td>
</tr>
<tr>
<td>3</td>
<td>3.7</td>
<td>5.06</td>
<td>Metabolite 17 -Glucuronide of M6</td>
</tr>
<tr>
<td>4</td>
<td>4.3</td>
<td>4.56</td>
<td>Metabolite 14 - Glucuronide of M5</td>
</tr>
<tr>
<td>5</td>
<td>5.8</td>
<td>6.43</td>
<td>Metabolite 8 - Glucuronide of M7</td>
</tr>
<tr>
<td>6</td>
<td>7.8</td>
<td>2.37</td>
<td>Metabolite 11 - Glucuronide of M1a</td>
</tr>
<tr>
<td>7</td>
<td>8.1</td>
<td>2.54</td>
<td>Metabolite 18 - Sulphate of M5</td>
</tr>
<tr>
<td>8</td>
<td>9.5</td>
<td>14.58</td>
<td>Metabolite 10 - Glucuronide of M1b</td>
</tr>
<tr>
<td>9</td>
<td>10.3</td>
<td>2.32</td>
<td>Metabolite 15 - Glucuronide of M12</td>
</tr>
<tr>
<td>10</td>
<td>13.2</td>
<td>4.06</td>
<td>Unknown</td>
</tr>
<tr>
<td>11</td>
<td>20.1</td>
<td>2.80</td>
<td>Metabolite 16 - Sulphate of M12</td>
</tr>
<tr>
<td>12</td>
<td>23.2</td>
<td>0.97</td>
<td>Metabolite 1a</td>
</tr>
<tr>
<td>13</td>
<td>24.9</td>
<td>0.41</td>
<td>Metabolite 1b Consistent with Reference Standard M1</td>
</tr>
</tbody>
</table>

Identified % of urinary radioactivity 92.41
Unidentified % of urinary radioactivity 7.59
Identified % of dose 49.44
Unidentified % of dose 4.06

M3 – Only observed in deconjugated samples as a trace component. The conjugate of M3 was not identified. M9 – Retention time 19 minutes. Trace component M13 – Retention time at 17 min. Trace component.
The principal nimesulide metabolite, 4'-hydroxy-nimesulide, was also measured in plasma and the kinetic data are consistent with metabolism of nimesulide to 4'-hydroxy-nimesulide being the major route of elimination for nimesulide. In combination the concentrations of nimesulide and 4'-hydroxy-nimesulide in plasma accounts for virtually all the radioactivity in plasma.

The distribution of radioactivity in excreta was consistent with previous data Bernareggi [5] with urine and faeces containing 62.5% and 36.2% of the administered dose respectively. A total of 16 metabolites of nimesulide were identified. At least 11 of these have been identified for the first time and these constitute a major proportion (>35-40%) of total drug that was metabolised. The pathways for biotransformation of nimesulide in man have been shown to proceed by 3 principle routes (Figure 4), namely cleavage of the molecule at the ether linkage, reduction of the NO₂ group to
NH₂ and ring hydroxylation. Cleavage of the ether linkage gave M6 which is conjugated with glucuronic acid to give M17. Reduction of the NO₂ group to NH₂ is proposed to produce the intermediate M2, which is hydroxylated to produce M3. M3 is then acetylated to produce M5. An alternative route is possible for the production of M5 in which M2 is acetylated to the postulated intermediate M4 which is in turn hydroxylated to give M5. The latter pathway is, however, less likely because the acetylation of the NH₂ group is thought to occur in the kidney and therefore is a terminal metabolic reaction. M5 is conjugated with sulphate and glucuronic acid to give M18 and M14, respectively. Ring hydroxylation of nimesulide gives the 4-hydroxy-derivative, M1. Conjugation of this molecule with sulphate gives M9. Conjugation of M1 with glucuronic acid gives M10, it is proposed that the principle position of hydroxylation is consistent with the reference standard M1. However, a second position of hydroxylation is proposed to give rise to a second step conjugate of molecular weight 500 (M11). M1 is also hydroxylated in a second position to give M12 which is then conjugated with glucuronic acid and sulphate to give M15 and M16, respectively.

The metabolites identified during these investigations are in agreement with the work of Carini et al. [8] but these authors only identified 5 metabolites of nimesulide in human urine (M1-M5). Metabolites M1, M2 and M5 were confirmed during the present study but M3 and M4 were not detected. These metabolites were previously reported as being present at very low concentrations. As a consequence they are proposed as intermediates in the biotransformation pathway. Additional phase 1 metabolites (M6, M7 and M12) have been identified which were not previously detected. The structural assignments of M6 and M7 and their glucuronide conjugates were confirmed with authentic reference standards. Carini et al. [8] also reported that the greater portion of an oral administration of nimesulide was excreted as conjugated metabolites. This was based on the extractability of metabolites before and after the enzymatic hydrolysis of urine and this has been confirmed in the present study by isolating conjugated metabolites from urine and analysing these metabolites by LC-MS/MS. This approach confirmed the presence of glucuronide and sulphate conjugates. Beta-glucuronidase from E. coli, which does not have any sulphatase activity, was used to hydrolyse the glucuronide conjugates present in urine. This allowed the confirmation of glucuronide conjugates identified by LC-MS/MS and also allowed the sulphate conjugates to be clearly visible in chromatograms of extracts of hydrolysed urine. Thus, the present experiments enable identification of the biotransformation pathway for nimesulide in man to be extended from 5 metabolites to 16 metabolites and 2 postulated intermediates. Greater than 92.4% of the urinary (0-24 h) radioactivity was accounted for by characterised metabolites. Thus, the most comprehensive metabolic pathway of nimesulide has now been obtained as a consequence of careful extraction isolation and mass spectrometric analytical procedures.

From the point of view of likely involvement of the nimesulide metabolites those thought to be involved with the development of hepatotoxicity from the drug include nitroso- and glucuronide metabolites [6,8-14,16-18,22]. Studies by Li et al. [15] indicate that two diimine metabolites of nimesulide may be formed by cytochrome P450 2C19 or 1A2 oxidation of the two 4-amino-metabolites, M3 and the N-de-acetylated form of M5. These postulated diimine reactive metabolites may act in forming adducts in a manner similar to that suggested with diclofenac [25,26] and paracetamol [27]. However, the formation of the N-deacetylated derivatives of the 4-amino-metabolites of nimesulide (Figure 4) would be a metabolic means of negating the hepatotoxic impact of these diimine metabolites. The relative proportions of the 4-amino metabolites that are formed in vivo are small in comparison with the total number and rate of formation of the metabolites of the drug (Table 3). The glucuronide metabolites may also represent another means of forming adducts [11] analogous to that seen with many other NSAIDs [27]. These and other reactive intermediates or metabolites including those with effects on mitochondrial functions and superoxide generation could also be candidates for hepatic injury by nimesulide [9,13,14,17,27,28]. Further quantification of the putative reactive metabolites along with studies on their mode of hepatic reactions may give insight into the relative contributions of these different metabolites in the development of hepatotoxicity form nimesulide.

Acknowledgments

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Declaration

The manuscript was prepared entirely by the authors who are responsible for its content.

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stress activates Nrf2 in human hepatocytes and mice but is not sufficient to induce hepatotoxicity in Nrf2-deficient mice. Chem Res Toxicol 23: 967-976.


