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Extractionless determination of 6-methoxy-2-naphthylacetic acid, a major metabolite of nabumetone, in human plasma by high-performance liquid chromatography

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Abstract

Following oral administration of the prodrug nabumetone, the major metabolite 6-methoxy-2-naphthylacetic acid (6-MNA) was determined in human plasma. Minimal sample preparation was followed by reversed-phase liquid chromatography and UV detection, affording high sample throughput. The lower limit of quantification (LLOQ) was 70 ng/ml, at a signal-to-noise ratio of 8:1. The assay method displayed good correlation ($r=0.997$), and can be readily employed in pharmacokinetic and bioequivalence studies.

Keywords: 6-Methoxy-2-naphthylacetic acid; Nabumetone

1. Introduction

Nabumetone is a relatively new non-steroidal anti-inflammatory drug (NSAID) which has proved effective in the treatment of rheumatoid and osteoarthritis. This prodrug undergoes extensive first-pass metabolism, yielding 6-MNA, which is believed to be largely responsible for the pharmacological activity [1]. Relatively few HPLC methods have been described for the determination of nabumetone and/or 6-MNA in human plasma [2–5].

Jang et al. [3] and Ray et al. [5] both described liquid–liquid extractions, following acidification of drug-containing plasma. Jang et al. acidified using a of combination 0.1 *M* HCl and 0.5 *M* citrate buffer (pH 3), while Ray et al. used 1.5 *M* HCl only. Both authors then extracted the analyte into an organic phase [ether and *n*-hexane–ethyl acetate (50:50), respectively] and evaporated to dryness under a gentle stream of nitrogen, after which the samples were reconstituted and injected onto normal-phase HPLC columns. While Jang et al. detected fluorometrically (excitation=284 nm, emission=320 nm), Ray et al. made use of UV detection at 280 nm, fluorometric detection having a higher sensitivity (0.1 μg/ml). The extraction procedure described by Al-Momani et al. [2] appears to be loosely based on the method described by Ray et al., while using UV detection (270 nm). Jang and Al-Momani used approximately 1 ml of serum/plasma, while Ray et al. used 0.5 ml of plasma.

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The present paper describes an HPLC assay of 6-MNA in which samples are prepared by protein precipitation, using naproxen as internal standard. Since a protein precipitation assay method has not yet been described, there is good motivation for developing such a procedure, considering the large number of samples generated by bioequivalence studies. The present paper describes a procedure that is well suited to rapid sample processing, requiring only a small volume of plasma (200 μl). 6-MNA was separated from the internal standard and endogenous plasma components using a Hewlett-Packard LiCrospher® 100 RP8 (5 μm) stainless-steel column (Hewlett-Packard, Palo, Alto, CA, USA), fitted with an Upchurch guard column, dry filled with Perisorb® RP-18 pellicular packing (Upchurch, WA, USA).

This assay method was used to quantify samples that were generated during a single-dose, two-way cross-over study with a wash out period of 14 days between 2 clinic days, following a 1000 mg oral dose of nabumetone. The study was designed to ascertain oral bioavailability between the reference product (Relifex®), and an as yet unnamed test product. The study was conducted on 30 healthy subjects (14 males and 16 females), aged between 19 and 29 years.

Venous blood samples were collected in heparinised glass tubes just prior to dosing and 1.5, 3, 4, 5, 6, 7, 8, 10, 12, 14, 18, 24, 48, 72, 96, 120 and 144 h thereafter. All samples were immediately handled on ice and centrifuged at 1200 *g* at 4°C for 10 min within 1 h of collection. Plasma was then transferred into sample tubes and stored at −20°C until analysis.

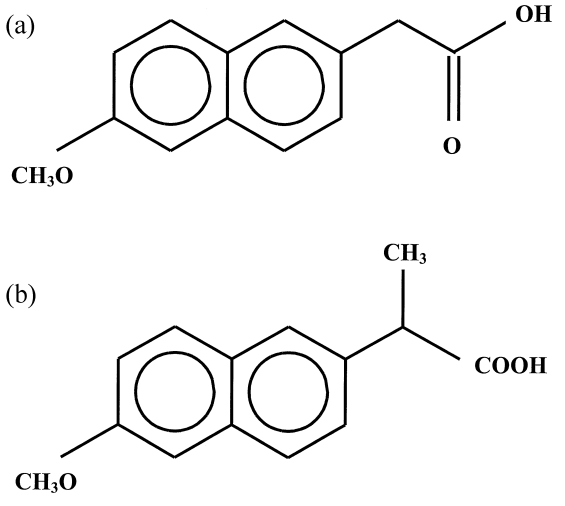

Fig. 1. (a) 6-MNA, (b) naproxen.

2. Experimental

2.1. Reagents and materials

6-MNA (Fig. 1a) was supplied by Wessex Fine Chemicals Ltd. (Billingshurst, West Sussex, UK) and naproxen (Fig. 1b) obtained from Syntex Inc. (Palo, Alto, CA, USA). Acetonitrile and methanol (B & J Brand™) were obtained from Baxter (Muskegon, MI, USA). Orthophosphoric acid (85%) and disodium hydrogen phosphate were obtained from Merck (Darmstadt, Germany). Citric acid (Fluka, Buchs, Switzerland) and 1-heptane sulphonic acid (Saarchem, Krugersdorp, South Africa) were used without further purification. All water used was purified by RO 20SA reverse osmosis system and Milli-Q® polishing system (Millipore, Bedford, MA, USA).

2.2. Apparatus

UV detection was performed by a Hewlett-Packard series 1100 variable wavelength detector monitoring at 280 nm. Separation was achieved on a LiCrospher® 100 RP8 (5 μm) stainless-steel column (Hewlett-Packard). The mobile phase consisted of acetonitrile–citric acid buffer (450:550, 20 m*M*, pH 2.8). 1-Heptane sulphonic acid (300 mg/l) was added and the apparent pH of the mobile phase adjusted to 3.1 using concentrated orthophosphoric acid. Mobile phase was delivered by a series 1100 isocratic pump (Hewlett-Packard, Palo, Alto, CA, USA) at 1.0 ml/min and at ambient temperature. A Hewlett-Packard series 1100 autosampler injected 10 μl onto the HPLC column. High speed centrifuging of microfuge tubes (Eppendorf, Hamburg, Germany) was done in a centrifuge 5416 (Eppendorf).

2.3. Preparation of calibration standards

6-MNA (5.091 mg) was weighed and directly dissolved in 36.06 g of blank plasma, contained in a

stoppered Erlenmeyer flask. This calibration standard was then shaken for 2 h using a GFL sample shaker (Bugwedel, Germany). This produced the highest calibration standard (145 μg/ml). Plasma dilution (1:1) of this calibration standard and subsequent standards yielded a total of 12 calibration standards, spanning a concentration range of 0.070–145 μg/ml.

Upper range quality controls (54.6, 30.4 and 17.3 μg/ml) were prepared in a similar fashion by dissolving 3.901 mg of 6-MNA in 73.40 g of blank plasma and preparing two serial dilutions from this highest quality control. The lower range quality controls were prepared by dissolving 2.472 mg of 6-MNA in 11.126 g of methanol and spiking 100 μl of this solution into 109.10 g of blank plasma. This quality control (0.330 μg/ml) was then serially diluted with plasma to produce the remaining quality controls (0.248, 0.165 and 0.083 μg/ml). The calibration standards and quality controls were stored at −20°C until assayed. Sufficient calibration standards and quality controls were prepared to develop and validate the assay method, as well as to assay all study samples. A new calibration line, along with quality controls, was included in each assay batch.

2.4. Sample preparation

Plasma (200 μl) was pipetted into a microfuge tube and an equal volume of acetonitrile containing naproxen (80 μg/ml) added. The microfuge tube was then sealed, vigorously vortexed (1 min), and centrifuged (6800 *g*, 5 min). An aliquot of 10 μl of the supernatant layer was injected onto the HPLC column.

3. Results and discussion

3.1. Sample preparation

Due to the large number of samples generated during comparative bioequivalence studies, it was thought beneficial to investigate extractionless sample preparation as an alternative to liquid–liquid and solid-phase extraction, with a view to maximising sample throughput. Furthermore, plasma concentrations following a single 1000-mg oral dose are appreciable [1] and it was speculated that the loss in sensitivity, traded off against rapid sample handling, would not be significant enough to render protein precipitation inappropriate for single-dose bioequivalence studies.

3.2. Method validation

Based on the ratio between analyte and internal standard peak height, the calibration line was found to be linear over the range 0.070–145 μg/ml and was characterised by the equation $y=(0.017 \text{ to } 0.021)x+(-0.0006 \text{ to } 0.0003)$, with a mean calibration curve slope C.V. of 6.2% ($n=15$) over a period of 3 weeks when using a $1/\text{concentration}^2$ weighting.

3.3. Chromatography

Analyte and internal standard were well separated from endogenous plasma components (Fig. 2), affording good chromatographic results.

3.4. Recovery

The analyte was fully recovered (~100%) in the supernatant layer, and the internal standard recovery was found to be 70%. Analyte recovery was determined in five-fold at high (30.4 μg/ml), medium (0.330 μg/ml) and low (0.083 μg/ml) concentration.

3.5. Sensitivity

The LLOQ was set at 70 ng/ml at a signal-to-noise ratio of 8:1, which was sufficient to detect 6-MNA in human plasma up to 144 h ($6\times t_{1/2}$), following a single 1000-mg dose of nabumetone. Over a time period of 144 h in which study samples were collected and assayed during a bioequivalence study, no sample was found to have a 6-MNA concentration below this LLOQ.

3.6. Inter and intra-day accuracy and precision

The intra- and inter-day accuracy and precision of the assay method (Tables 1 and 2) showed the assay method to be robust over the 21 days in which 15 batches, each consisting of 72 samples, 10 cali-

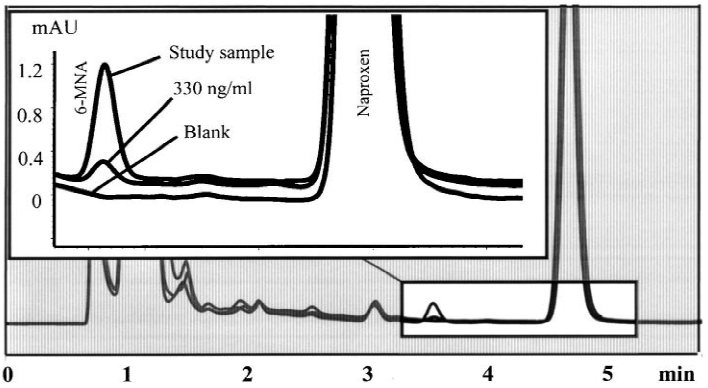

Fig. 2. Enlargement of overlaid chromatograms, showing good resolution between 6-MNA and endogenous plasma components. The study sample (96 h after the ingestion of 1000 mg of nambumetone) produces a peak roughly five-times larger than a 330 ng/ml calibration standard.

Table 1
Intra-day accuracy and precision of quality controls ($n=5$)

Nominal concentration (ng/ml)	Found concentration (ng/ml)	n	C.V. (%)
30 400	30 400	5	2.7
17 300	17 300	5	2.9
330	334	5	4.1
248	254	5	6.5
165	165	5	2.3
83.0	91.0	5	4.7

bration standards and 8 quality controls were processed.

3.7. Matrix stability

Calibration standard and quality control response factors were monitored daily and compared to freshly prepared standard solutions of 6-MNA in the mobile phase. 6-MNA was found to be stable in human plasma for at least 3 weeks when stored at −20°C.

Table 2
Inter-day accuracy and precision of assay batch quality controls, completed over 21 days

Nominal concentration (ng/ml)	Found concentration (ng/ml)	n	C.V. (%)
30 400	30 220	30	3.2
17 300	16 890	30	4.0
330	322	29	5.8
165	154	15	7.5
83.0	79.6	13	6.5

3.8. Specificity

Plasma from six different sources was extracted and no peak was found to co-elute with 6-MNA or the internal standard. In addition, pre-dosing samples from the 30 subjects assayed were included in the batches and no interference was observed.

3.9. On-instrument stability

6-MNA was found to be stable in the final reconstituted solution for the period during which samples were on the instrument (approximately 10 h).

3.10. Application

This assay method was employed in a single-dose bioequivalence study of two film-coated oral formulations of nabumetone. Good chromatographic results were obtained in spite of minimal sample preparation. There was good concordance between the calculated parameters and existing literature [1], as reflected in Table 3.

Table 3
Comparison between experimentally determined pharmacokinetic data and existing literature

Reference	*n*	Dose (mg)	C_{max} (μg/ml)	T_{max} (h)	$t_{1/2}$ (h)	AUC(0–∞) (μg h/ml)
Relifex®	29	1000	25.1 (10.1–38.5)	24.0 (1.5–48.0)	22.8 (13.8–32.1)	1444 (537–2436)
Test product	29	1000	26.5 (10.3–46.3)	10.0 (3.0–24.0)	23.1 (16.0–33.1)	1412 (513–2213)
Kendall et al. [1]	12	1000	21.9	13	26.3	1120

3.11. Calculation of pharmacokinetic variables

The maximum concentration (C_{max}) and time to maximum concentration (T_{max}) were read directly from the observed concentrations. The apparent terminal half-life ($t_{1/2}$) was calculated by fitting the concentration versus time data of the terminal phase to a single exponential function (Ce^{-kt}) using sum of least squares regression analysis. The terminal half-life was then calculated as $t_{1/2}=0.693/k$, where k is the terminal rate constant. The area under the plasma concentration versus time data, extrapolated to infinity [AUC(0–∞)], was calculated by adding $C(t_{last})/k$ (the plasma concentration at the last sampling time divided by the terminal rate constant) to the total area beneath the plasma concentration versus time curve at the last sampling time (AUC(0–t_{last})). Thus $AUC(0-\infty) = AUC(0 - t_{last}) + C(t_{last})/k$. The mean profiles (Fig. 3) display good agreement between the test and reference product. The financial viability of processing all the samples generated during the study was first assessed by the comparison of pooled samples from each treatment phase [6], producing mean treatment profiles. On the strength of the pooled plasma results, samples were individually processed. The mean 6-MNA concentration versus time profiles (Fig. 3) compare well with those reported by Kendall et al. [1]

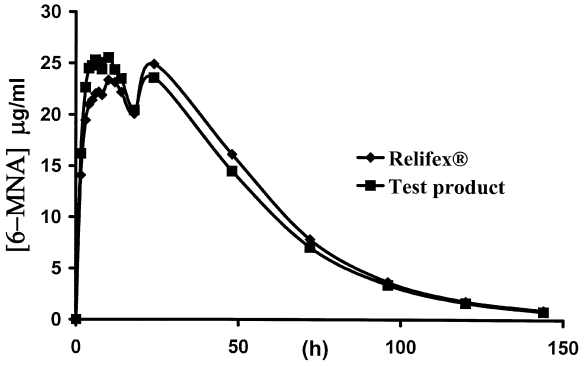

Fig. 3. Mean profiles of the test and reference product (Relifex), following a 1000-mg oral dose of nabumetone.

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