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An LC–MS/MS procedure for the quantification of naproxen in human plasma: Development, validation, comparison with other methods, and application to a pharmacokinetic study

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ABSTRACT

A sensitive, precise and accurate quantitative LC–MS/MS method for the measurement of naproxen in human plasma was developed and completely validated according to current FDA and EMA guidelines. The new method employs acetonitrile protein precipitation for sample preparation and uses ketoprofen as the internal standard. Suitability of the new assay was assessed in comparison with 36 reported bioanalytical assays and the pharmacokinetic results obtained by the new method were compared to 11 reported studies in humans. The principal advantage of this LC–MS/MS method is the simultaneous achievement of high absolute recovery (90.0 ± 3.6%), acceptable sensitivity (lower limit of quantitation of 0.100 μg/mL), high inter-day precision (CV ≤ 9.4%), high analytical recovery (between 94.4 and 103.1%), and excellent linearity over the concentration range 0.100–50.0 μg/mL ($r^2 \geq 0.998$) combined with a short run time of only 2 min.

1. Introduction

Naproxen, 2-(6-methoxynaphthalen-2-yl)propanoic acid, is a member of the aryl acetic acid group of non-steroidal anti-inflammatory drugs (NSAIDs), that has been used as an over-the-counter analgesic, anti-inflammatory and antipyretic agent for decades. It is also used to treat rheumatoid arthritis and other inflammatory rheumatic diseases. Its mechanism of action has just recently been elucidated [1–3]. Naproxen is administered exclusively as the pharmacologically active *S*-enantiomer [4], which is formulated either as the free acid or as the corresponding sodium salt, that in turn leads to a more rapid absorption from the gastrointestinal tract [5,6]. The mechanism of action of naproxen, like that of other NSAIDs, relies on the inhibition of cyclooxygenase (COX) activity, which was first proposed by Vane in 1971 [7]. Today it is known that COX exists in at least two isoforms, COX-1 and COX-2 [8]. COX-1 catalyzes the biosynthesis of prostaglandins that are important for maintaining physiological functions, *e.g.* synthesis of prostacyclin, which in turn is cytoprotective when released by the gastric mucosa, or anti-thrombogenic when released by the vascular endothelium, whereas COX-2 produces prostaglandins, which are involved in pathophysiological processes like inflammation, fever and pain. Adverse effects, *e.g.* gastrointestinal bleeding and ulceration, associated with the use NSAIDs were found to be caused by the inhibition of the COX-1 isoenzyme [9] and led to the development of selective COX-2 inhibitors [10]. Rofecoxib, a selective COX-2 inhibitor, was withdrawn from the market in 2004 as a clinical trial comparing naproxen and rofecoxib showed fewer serious gastrointestinal adverse events, but an increased risk of heart attack [11]. However, recognition of new avenues for selective COX-2 inhibitors such as cancer, Alzheimer's disease, Parkinson's disease, schizophrenia, major depression, ischemic brain injury and diabetic peripheral nephropathy has lead to a new the interest selective COX-2 inhibitors [12].

In view of the discussion on the adverse effects of NSAIDs in general [13], it is crucial that for comparative pharmacokinetic/pharmacodynamic investigations of NSAIDs, which may help to understand the risks of any individual NSAID better, bioanalytical methods must be available that meet today's criteria for a reliable drug assay. This can usually be achieved by use of modern bioanalytical technologies like LC–MS/MS and meeting the requirements of FDA's and EMA's bioanalytical guidelines [14–16].

Naproxen may be administered as a suppository formulation or orally in form of a suspension or tablet. When administered orally

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at therapeutic doses, naproxen is completely absorbed with peak plasma concentrations being reached within 2 h [17,18] and more than 99% bound to albumin [19]. Naproxen is extensively metabolized by phase I metabolism to 6-*O*-desmethylnaproxen and both, naproxen and 6-*O*-desmethylnaproxen, are conjugated by phase II metabolism to glucuronic acid and sulfate giving rise to five possible conjugates [20].

The aim of the present study was to develop and validate a sufficiently sensitive, precise, accurate and very fast LC–MS/MS (SRM) method for the quantification of unchanged naproxen in human plasma to be applied in a pharmacokinetic study to show the reliability of the assay in samples from humans, who received an oral dose of 220 mg naproxen sodium. The superiority of the new assay for its intended use in human pharmacokinetic studies was to be corroborated by an extensive comparison of its validation and application data to published methods of naproxen quantitation.

2. Experimental

2.1. Chemicals and reagents

Naproxen sodium, chemical purity: 99.95%, and ketoprofen, chemical purity: 99.55%, were obtained from Sigma–Aldrich (Munich, Germany). Acetonitrile was of HPLC-grade and from FSA Laboratory Supplies (Loughborough, UK). Other chemicals used were of analytical grade and from VWR International (Darmstadt, Germany). Ultra pure water was obtained using a Milli-Q purification system from Millipore Corporation (Bedford, MA, USA).

2.2. Plasma samples

Drug-free human plasma containing heparin as the anticoagulant was used for method validation. Hemolyzed plasma was prepared by adding 1% of frozen and thawed drug-free blood to drug-free human plasma using heparin as the anticoagulant.

2.3. Calibration standards and spiked quality control standards

For validation of the naproxen method and for calibration of human plasma measurements a human plasma calibration curve of 11 standards, including a blank sample, which was not used for calculation of linear regression, was prepared every validation day and before starting the measurement of study samples. Spiked quality control standards (SQC) were prepared for method validation on each validation day in order to control the precision and accuracy of the assay during the measurement of study samples. On each validation day a stock solution of 5 mg/mL naproxen in methanol (stock solution I) was used to prepare the calibration standards and another stock solution of 5 mg/mL naproxen in methanol (stock solution II) was used to prepare the SQC samples. Calibration standards (L1–L10) in the theoretical concentrations 50.0, 45.0, 25.0, 12.5, 5.00, 2.50, 1.00, 0.400, 0.200 and 0.100 μg/mL of naproxen in human plasma, as well as SQC samples in the theoretical concentrations 50.0, 25.0, 3.00, 0.300 and 0.100 μg/mL of naproxen in human plasma were prepared on each day of analysis by serial dilution.

2.4. Sample preparation

All thawing of frozen plasma samples was done in a water bath. All pipetting steps were carried out using the Multimek™ Automated 96-Channel Pipettor from Beckmann Coulter GmbH (Unterschleißheim, Germany). Human plasma samples (100 μL) were deproteinized by addition of 200 μL of acetonitrile containing the internal standard (ketoprofen, 2 μg/mL). After thorough mixing the samples were centrifuged for 10 min at 3000 rpm (2733 × *g*) at approximately +4 °C. 40 μL of the supernatant was mixed with 360 μL 0.02 M ammonium acetate buffer (pH 4.0). After mixing, 10 μL of each sample were injected onto the LC–MS/MS system.

2.5. Liquid chromatography/mass spectrometry

The liquid chromatography system consisted of a L-6200A HPLC pump from Merck KGaA (Darmstadt, Germany) and a CTC Combi Pal Autosampler from CTC Analytics (Zwingen, Switzerland). Chromatographic separations were performed on a Betasil C18, 5 μm (50 mm × 4.6 mm i.d.) column from Thermo Electron Corporation (Dreieich, Germany). The mobile phase, delivered at a flow rate of 1.0 mL/min at ambient temperature, consisted of 0.02 M ammonium acetate buffer (pH 4.0) and acetonitrile (30/70,v/v). 10 μL of sample was injected.

The detection was performed using a AB SCIEX API 3000™ triple quadrupole mass spectrometer fitted with a heated nebulizer interface, both from AB SCIEX (Concord, Ontario, Canada). High purity nitrogen gas was used as nebulizer, curtain, auxiliary, and collision gas. The mass spectrometer was operated in negative ion mode using the following settings: nebulizer current (NC): −3 μA, probe temperature: 500 °C, orifice voltage (OR): −6 V, collision energy: 10 eV. Quantification was performed using selected reaction monitoring (SRM) of the transitions m/z 229 → m/z 185 and m/z 253 → m/z 209 for naproxen and the internal standard ketoprofen, respectively. The dwell time per SRM transition was 175 ms with a pause time of 2 ms.

2.6. Data acquisition and processing

Data acquisition was performed with Sample Control version 1.4 from AB SCIEX (Concord, Ontario, Canada, 1993–1998). Data processing was performed with LC2Tune version 1.4 from AB SCIEX (Concord, Ontario, Canada, 1991–1998) and with MacQuan version 1.6 from AB SCIEX (Concord, Ontario, Canada, 1991–1998). All of these software products were supplied by AB SCIEX Germany GmbH (Darmstadt, Germany). Calculations were performed with Microsoft Excel 2000 from Microsoft Co. (Redmond, WA, USA, 1985–2000).

2.7. Validation procedure

The method was validated according to the most recent US Food and Drug Administration (FDA) guidelines [14]. Specificity, linearity, lower limit of quantification (LLOQ), inter-day and intra-day precision and accuracy as well as absolute recovery and stability of naproxen were evaluated. Moreover the influence of hemolyzed plasma, the influence of six different batches of human plasma and the influence of 1:5 dilution on the determination of naproxen and matrix effect were studied.

2.7.1. Determination of specificity

The specificity of the naproxen method was determined by screening six different batches of control drug-free human plasma. The samples were prepared as previously described with and without addition of the internal standard. There should be no co-eluting peaks with areas of more than 20% of the analyte peak area at the LLOQ at or near the retention time of naproxen.

2.7.2. Evaluation of linearity, sensitivity, inter-day and intra-day precision and accuracy and low limit of quantitation

For the determination of linearity, sensitivity, inter-day precision and accuracy as well as lower limit of quantitation (LLOQ) of the naproxen assay a calibration curve of eleven standards, including a blank sample which was not used for linear regression, and five sets of SQC samples were prepared and analyzed for naproxen

in human plasma on each of five consecutive days. The calibration curves were evaluated individually by linear regression and the concentrations of the calibration standards were back-calculated. The slopes, intercepts and the correlation coefficients of the corresponding individual curves were calculated. The calibration curves were accepted if there were not more than two outliers. If there were two outliers they had not to be adjacent. A calibration standard was defined as an outlier if the back-calculated concentration deviated more than 15% from the theoretical concentration at all concentrations except for the lowest concentration, where a deviation of less than 20% was accepted. Means, standard deviations, coefficients of variation (%) and accuracy (%) were calculated for the back-calculated concentrations of each calibration standard. The spiked SQC samples were calculated by the corresponding calibration curve. The LLOQ, defined as the lowest SQC concentration at which both inter-day precision and accuracy were less than or equal to 20%, was evaluated. For the evaluation of the intra-day precision and accuracy of the assay, five sets of spiked SQC samples of naproxen in human plasma were analyzed on a validation day. The concentrations were calculated by the corresponding calibration curve. Means, standard deviations, coefficients of variation (%) and accuracy (%) were calculated for each SQC. All calculations were performed without rounding and results were rounded to three significant digits. The accuracy and precision were determined as accuracy (%) and coefficient of variation (CV, %) respectively and as follows: CV (%) = (standard deviation/mean) × 100, accuracy (%) = mean assayed concentration × 100/theoretical conc. The inter- and intra-day CVs for the spiked quality control samples as well as the accuracies should be within ±15% except at the LLOQ where a value of ±20% is accepted.

2.7.3. Signal to noise

On the first 5 validation days, the signal to noise ratio was determined for naproxen at the LLOQ (0.100 μg/mL). The mean value should be greater or equal to 5.

2.7.4. Determination of absolute recovery of naproxen and the internal standard in human plasma

For the determination of recovery of naproxen and ketoprofen (IS), five spiked quality control standards in human plasma (SQC1–SQC5) and five spiked quality controls in processed blank human plasma (QBL1–QBL5) were prepared. Each SQC and each QBL was measured five times. The recovery for the analytes (naproxen and internal standard) was evaluated according to the equation: recovery (%) = (mean peak area of analyte in spiked and processed human plasma samples/mean peak area of analyte added to processed blank human plasma) × 100.

2.7.5. Evaluation of stability

Stability of naproxen in human plasma was assessed by analyzing five SQC samples at two different concentrations (SQC1 and SQC4), exposed to different conditions of time and temperature. The results were compared with those for SQC samples prepared freshly. The short-term stability was evaluated after exposure of the plasma samples to room temperature for 2 h and 4 h. The long-term stability was assessed after storage of the test samples at −20 °C and −70 °C. Of each group five of those SQC samples were measured 2 days, 4 days and 3 weeks after the start of the stability test. The freeze/thaw stability was determined after three freeze/thaw cycles −70 °C to room temperature on consecutive days. The samples were frozen at −70 °C for 24 h and thawed at room temperature. After complete thawing the samples were refrozen at −70 °C for 12–24 h at −70 °C. This step was repeated two times. After the third thawing the samples were analyzed. The post-preparative storage stability of naproxen was assessed at approximately +4 °C (autosampler temperature) and approximately −70 °C for 24, 48, and 72 h after preparation. The stability of stock solutions of naproxen and of the internal standard was also tested at room temperature for 6 h. The stock solutions standing for 6 h at room temperature were analyzed and compared with stock solutions that were used directly five times. Statistical evaluation was performed by calculating 95% ANOVA based confidence intervals for the ratios between the concentrations measured after given periods of time or after repeated thawing/freezing and the respective control (to allow for any contribution of assay imprecision). Instability was concluded if both the upper and lower limit of the confidence interval were greater than −10%.

2.7.6. Influence of hemolyzed plasma on the determination of naproxen

On a validation day the influence of hemolyzed plasma on the determination of naproxen was determined by measuring five samples of spiked quality control standard no. 1 and 4 in hemolyzed human plasma. The hemolyzed human plasma was processed as previously described for unhemolyzed human plasma. The spiked quality control standards in hemolyzed human plasma were analyzed together in the same run with the spiked quality control standards prepared in unhemolyzed human plasma. The CV should be ≤15%. The mean accuracy should be within ±15% of the nominal value.

2.7.7. Influence of different batches of human plasma on the determination of naproxen

On a validation day the influence of six different batches of human plasma on the determination of naproxen was investigated by measuring three samples of each spiked quality control standard nos. 1 and 4 in six different batches of human plasma. For each SQC concentration, the mean value, standard deviation and CV were calculated. The CV should be ≤15%. The mean accuracy should be within ±15% of the nominal value.

2.7.8. Influence of dilution on the determination of naproxen in human plasma

The influence of a 1:5 dilution on the determination of naproxen in human plasma was determined by measuring five samples of spiked quality control standard SQC D5 in human plasma which was at first diluted 1:5 with drug-free human plasma and then processed as previously described. For the diluted samples, mean, standard deviation and CV were calculated. The CV should be ≤15%. The mean accuracy should be within ±15% of the nominal value.

2.7.9. Matrix effect

The matrix effect was investigated with quantitative determination of the matrix factor (MF). To determine the MF for naproxen the analyte and ketoprofen were added to the mobile phase and drug free processed blank human plasma samples. Each sample was measured fivefold. The area ratios of naproxen/ketoprofen were calculated in processed blank human plasma and the mobile phase. The MF was calculated by the following equation: MF = mean area ratio naproxen in processed blank human plasma/mean ratio of naproxen in the mobile phase. The variability of the matrix factor, as measured by the CV, should be less than 15%. A matrix factor greater or smaller than one suggests analyte ion enhancement or suppression due to matrix components. A value of one signifies no matrix effect.

2.8. Assay suitability study

The suitability of the LC–MS/MS procedure developed and validated here was investigated in a pharmacokinetic study of naproxen, where a tablet of 220 mg naproxen sodium was administered orally in the fasting state in the morning of the study day.

Blood collection was performed immediately before administration and at 0.167, 0.333, 0.500, 0.667, 0.833, 1.00, 1.25, 1.50, 1.75, 2.00, 2.50, 3.00, 4.00, 6.00, 8.00, 10.0, 12.0, 16.0, 24.0, 48.0 and 72.0 h after administration. The subject samples (in randomized order) were measured together with 11 calibration standards and 16 spiked quality control standards (SQC1 = 40.4 μg/mL, SQC2 = 20.2 μg/mL, SQC3 = 2.43 μg/mL and SQC4 = 0.243 μg/mL) within 6 h. The determined concentrations of the spiked quality control standards were compared to the theoretical concentrations for accuracy.

2.9. Pharmacokinetic analysis

The pharmacokinetic parameters were calculated as follows: the area under the curve from time zero to the time of the last quantifiable concentration ($AUC_{0\rightarrow t}$) was calculated using the linear trapezoidal rule. The area under the curve from time zero to infinite ($AUC_{0\rightarrow\infty}$) was calculated as: $AUC_{0\rightarrow t} + C_t/K_{el}$, where: C_t = the last observed quantifiable concentration for that treatment and K_{el} = the elimination rate constant. To calculate the elimination rate constant (K_{el}), regression analyses were performed on the natural log (ln) of plasma concentration values (y) versus time (x). Calculations were made between a time point where log-linear elimination phase begins (TLIN) and the time at which the last concentration above the limit of quantitation occurred. The K_{el} was taken as the slope multiplied by −1 and the apparent half-life ($T_{1/2\,el}$) as $(\ln 2)/K_{el}$. TLIN, the time point where ln-linear K_{el} calculation begins, and LQCT, the sampling time of the last quantifiable concentration used to estimate the K_{el}, were determined for each subject and for each treatment. At least four non-zero observations during the terminal elimination phase were used to calculate the K_{el}. A minimum of 3 observations were used if less than 4 observations were available.

3. Results and discussion

3.1. Full scan and product ion spectra of naproxen and ketoprofen

A mass spectrum of naproxen was recorded in the negative ion mode in the first quadrupole (Q1) of the API 3000™ mass spectrometer by continuous infusion of a solution containing approximately 10 μg/mL of naproxen in mobile phase. The peak in the mass spectrum at m/z 229 corresponds to the deprotonated molecule $[M-H]^-$ (Fig. 1 A). The most abundant ion in the product ion spectrum of naproxen was m/z 185, which was selected for the SRM transition and corresponds to the loss of carbon dioxide (44 u) from the deprotonated molecule (Fig. 1B). Furthermore, a mass spectrum of ketoprofen (IS) was recorded under the same conditions (Fig. 2A). The most abundant ion in the product ion spectrum of ketoprofen was m/z 209, which was selected for the SRM transition and corresponds to the loss of carbon dioxide (44 u) from the deprotonated molecule (Fig. 2B). Thus the mass spectrometer was set as follows: m/z 229 for naproxen and m/z 253 for ketoprofen (IS) as precursor ions, and m/z 185 for naproxen and m/z 209 for the internal standard as product ions in the SRM mode. Structures of precursor ions and generated product ions corresponding to the detected m/z ion fragments are shown in Figs. 1B and 2B.

3.2. Validation

The present study was conducted in accordance with the FDA guidances for industry (bioanalytical method validation, bioavailability and bioequivalence studies [14,15]), and the EMA guideline on validation of bioanalytical methods [16], which provide assistance in developing bioanalytical method validation information to yield reliable results that can be safely interpreted in human clinical pharmacology, pharmacokinetic, bioavailability,

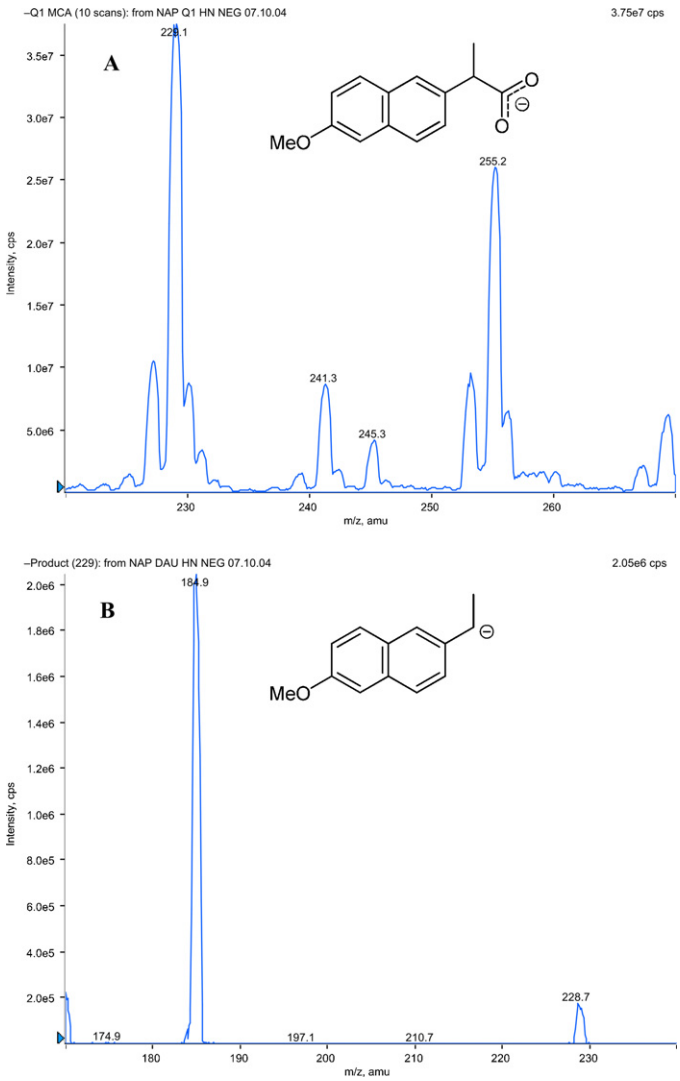

Fig. 1. Full-scan (A) and product ion mass spectra (B) of naproxen.

PK/PD-relationship and bioequivalence studies requiring pharmacokinetic evaluation. The present developed and validated LC–MS/MS method meets all criteria suggested by the guidance.

3.2.1. Assay specificity

Based on the analysis of drug-free human plasma (n = 6), endogeneous matrix components did not interfere with naproxen and the internal standard at their retention times and over the concentration range described herein. Fig. 3 shows typical SRM chromatograms for a blank plasma sample, a blank plasma sample spiked with naproxen (at LLOQ = 0.100 μg/mL) and ketoprofen (IS), a plasma sample from a patient 72 h after the administration of naproxen (naproxen concentration = 0.484 μg/mL) and a plasma sample from a patient 0.5 h after the administration of naproxen (naproxen concentration = 44.7 μg/mL).

3.2.2. Linearity of calibration curves, precision and accuracy of calibration standards and the LLOQ

The linear regression of the peak area ratios versus concentrations was fitted over the concentration range of 0.100–50.0 μg/mL for naproxen in human plasma. The mean linear regression equation of the calibration curves generated during the validation was $y = 0.002\ (\pm 0.001) + 0.073\ (\pm 0.004)\ x$, where y represents the ratio of naproxen peak area to that of the internal standard, and x

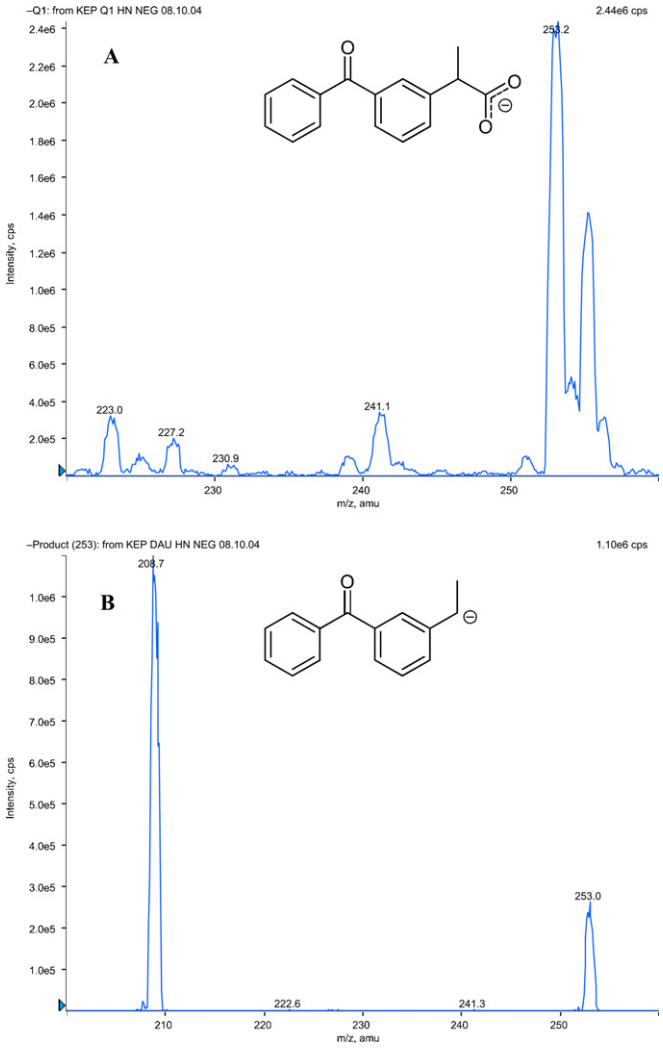

Fig. 2. Full-scan (A) and product ion mass spectra (B) of ketoprofen (IS).

represents the plasma concentration of naproxen. Good linearity was obtained in the validated concentration range. The correlation coefficients of the weighted calibration curves were ≥0.998. The inter-day precision of the back-calculated calibration standards ranged from 1.6 to 5.7% and the inter-day accuracy ranged from 97.1 to 105.0%. Using 100 μL of plasma the lower limit of quantification (LLOQ) for naproxen was 0.100 μg/mL, which was sufficient for the pharmacokinetic study conducted. The inter-day precision and accuracy obtained at the LLOQ were 3.5 and 101.6%, respectively.

3.2.3. Signal to noise

The signal to noise ratio of naproxen at the LLOQ (0.100 μg/mL) was >5 on all validation days.

3.2.4. Precision, accuracy and absolute recovery

Table 1 summarizes the intra- and inter-day precision and relative error for naproxen, evaluated by assaying the SQC samples. The inter-day precision of the spiked quality control samples for naproxen in human plasma ranged from 4.4 to 9.4% with an accuracy between 94.4 and 103.1%. The intra-day precision and accuracy of naproxen in human plasma ranged from 0.9 to 8.4% and from 97.5 to 105.2%, respectively. The obtained results were within the acceptance criteria of no more than ±20% deviation at LLOQ and no more than ±15% deviation for standards above LLOQ. The signal-to-noise value at the LLOQ was greater than 5 on all validation days.

The mean absolute recovery of naproxen over the whole concentration range was determined as 90 ± 3.6%. The mean absolute recovery of the internal standard (ketoprofen) at the working concentration was determined as 80.6 ± 1.3%. All recoveries had relative standard deviations better than 4.0% throughout the entire standard concentration ranges, showing good consistency. The simple one-step protein precipitation procedure showed satisfactory recovery.

3.2.5. Stability

The results of the 95% confidence interval calculation showed no evidence of instability during chromatography, precipitation and sample storage processes for naproxen in human plasma samples. Stability data are shown in Table 2. In addition, naproxen and the internal standard (ketoprofen) stock solutions were stable for at least 6 h at room temperature.

3.2.6. Influence of hemolyzed plasma on the determination of naproxen

No influence of hemolyzed human plasma on the accuracy and precision of the method was observed. The mean concentration values for hemolyzed plasma were within ±15% of the nominal value with mean accuracies of 102.3 and 102.2% at 50.0 and 0.300 μg/mL, respectively. The CVs for hemolyzed plasma at 50.0 and 0.300 μg/mL were 1.1 and 3.8%, respectively.

3.2.7. Influence of different batches of human plasma and 1:5 dilution on the determination of naproxen

No influence on the analysis of naproxen could be shown for six different batches of human plasma. The mean concentration values for the six different batches of human plasma were within ±15% of the nominal value with mean accuracies of 90.8 and 101.3% at 50.0 and 0.300 μg/mL, respectively. The CVs for the different batches of human plasma at 50.0 and 0.300 μg/mL were 3.5 and 6.7%, respectively.

No influence on the analysis of naproxen could be shown for a 1:5 dilution of human plasma samples. The mean concentration value was within ±15% of the nominal value with a mean accuracy of 94.6%. The CV for the 1:5 diluted human plasma samples at 40.4 μg/mL was 1.8%.

3.2.8. Matrix effect

The matrix factor was 1.072. This value indicates the absence of a matrix effect in the analytical system.

3.3. Comparison of current method with existing methods

Numerous methods have been used to analyze naproxen in a vast variety of matrices. The following will focus on a concise survey of methods reported to date for the analysis of naproxen in clinical samples, *e.g.* plasma, serum, and urine. A comparison of the present LC–MS/MS method will be included as appropriate.

As outlined in Table 3, different techniques have been applied for the analysis of naproxen, *i.e.* liquid chromatography with MS/MS (LC–MS/MS, 2 reports [21,22]), UV (LC-UV, 16 reports [21,23–37]) or fluorescence detection (LC-FLUO, 7 reports [20,23,38–42]), gas chromatography with MS (GC–MS, 2 reports [43,44]) or flame ionization detection (GC-FID, 2 reports [45,46]), spectrofluorimetry (FLUO, 3 reports [47–49]) and thin layer chromatography (TLC/HPTLC-UV/FLUO, 3 reports [50–52]). Furthermore, several less frequently used techniques like chip-based immunoaffinity capillary electrophoresis (IACE [53]), micellar electrokinetic capillary chromatography with laser-induced fluorescence detection (MECC-LIF [54]), liquid chromatography with electrogenerated chemiluminescence (LC-ECL [55]) or electrochemical detection

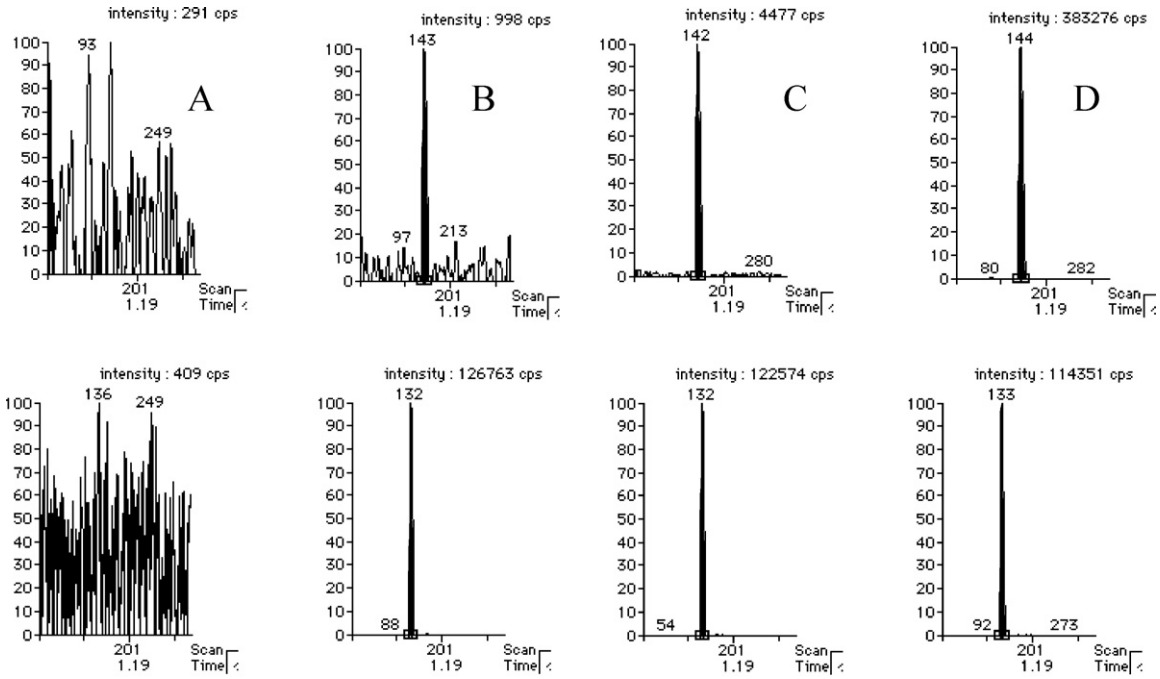

Fig. 3. Representative SRM chromatograms for naproxen (top) and ketoprofen (IS, bottom) in human plasma: (A) a blank plasma sample; (B) a blank plasma sample spiked with naproxen (at LLOQ = 0.100 μg/mL); (C) a plasma sample from a subject 72 h after the administration of naproxen (naproxen concentration = 0.484 μg/mL) and (D) a plasma sample from a subject 0.5 h after the administration of naproxen (naproxen concentration = 44.7 μg/mL).

(LC-EC [56]), and micelle stabilized room temperature phosphorescence (MS-RTP [57]) have also been applied.

Although many of these methods have been successfully applied, their use for analysis of high sample numbers remains limited by several drawbacks. Both, GC–MS and GC-FID require a suitable derivatization step due to the polar nature of naproxen, *e.g.* by use of *N*,*O*-bis(trimethylsilyl)acetamide [43], tetrabutylammonium hydroxide [44] or diazomethane [45,46], implying additional sample preparation time and costs. However, GC methods have not been used to quantitate naproxen in clinical samples since 1981.

Furthermore, sample preparation usually takes 80% of the total analysis time [58]. While more or less protein-free samples, *e.g.* urine or intestinal perfusate, may be used for LC analysis after simple filtration or centrifugation [24,55], protein-rich samples like serum and plasma must be freed of their protein content. This can be achieved by cost-intensive ultrafiltration [53], liquid–liquid extraction (LLEx, 23 reports [21,23,25–31,38–40,43–51,54]), protein precipitation (PP, 8 reports [20,22,32–34,41,52]) or solid-phase (micro)extraction (SP(M)E, 4 reports [35,36,42,56]). As liquid–liquid extraction may be time-consuming and thus unsuitable for the analysis of large batches, protein precipitation is a feasible alternative. While the main attraction to protein precipitation has been its speed, simplicity and universality, the less clean extracts obtained sometimes require more sophisticated chromatography [59]. However, the present LC–MS/MS method reduces the shortest run time reported to date involving protein precipitation [33,34,41] from 5 to 2 min without any interference from co-eluting analytes. As expected, samples prepared for liquid chromatography by solid-phase (micro)extraction provide very low limits of quantitation (LLOQs), *e.g.* 5 ng/mL by LC-UV [36] or 8 ng/mL by LC-FLUO [42], but analytical ranges in pharmacokinetic studies will usually spread from 5 to 120 μg/mL [40] and thus one might request a minimum LLOQ of 0.5 μg/mL. Since, solid-phase (micro)extraction might raise additional costs without giving analytical benefit to the analysis of clinical samples in pharmacokinetic studies protein precipitation by acetonitrile was chosen here.

Next to sample preparation short chromatography run times are pivotal to the analysis of high sample numbers. Until today, no liquid chromatography of naproxen has been reported with a run time of less than 5 min. More than 50% of the liquid chromatography based methods outlined in Table 3 report run times above 10 min (14 reports [21–24,27,28,31,32,35,36,38,40,44,56]). Due to UV and fluorescence detection, of which especially the former lacks additional analyte discrimination, extended run times are needed to achieve good chromatographic resolution and to avoid potential interferences from endogenous components, naproxen metabolites or co-administered drugs. Here, LC–MS and LC–MS/MS techniques are particularly useful to provide a specific and fast determination.

Sultan et al. [21] and Miksa et al. [22] described two methods to simultaneously quantify and identify naproxen and other NSAIDs in human plasma, employing LC–MS in full-scan MS

Table 1
Intra- and inter-day precision and accuracy for the analysis of naproxen in human plasma (five days, five replicates per day).

Theoretical concentrations of naproxen in human plasma (μg/mL)	Intra-day CV (%)	Intra-day accuracy (%)	Inter-day CV (%)	Inter-day accuracy (%)
SQC1 (50.0 μg/mL)	0.9	105.2	4.4	103.1
SQC2 (25.0 μg/mL)	2.5	103.0	4.8	101.5
SQC3 (3.00 μg/mL)	1.7	100.0	4.6	95.0
SQC4 (0.300 μg/mL)	5.7	97.5	4.5	94.4
SQC5 (0.100 μg/mL)	8.4	102.9	9.4	98.8

Table 2
Statistical evaluation of the stability experiments for naproxen. Instability was concluded if both the upper and the lower limit of the confidence interval were greater than −10%. No significant decrease was observed when compared to fresh samples.

Stability experiment	Concentration (μg/mL)	Time	Point estimator (%)	Lower limit (%)	Upper limit (%)
Short-term stability	SQC1 (50.0 μg/mL)	2 h	96.06	93.95	98.23
		4 h	97.11	95.63	98.62
	SQC4 (0.300 μg/mL)	2 h	103.82	98.96	108.91
		4 h	102.16	96.57	108.08
Post-preparative stability (autosampler temperature approximately 4 °C)	SQC1 (50.0 μg/mL)	24 h	94.62	93.34	95.92
		48 h	104.74	103.31	106.20
		72 h	107.10	105.61	108.61
	SQC4 (0.300 μg/mL)	24 h	95.88	90.40	101.69
		48 h	96.47	94.37	98.62
		72 h	100.07	97.70	102.49
Post-preparative stability (approximately −70 °C)	SQC1 (50.0 μg/mL)	24 h	93.35	92.53	94.17
		48 h	105.18	104.21	106.16
		72 h	106.66	104.93	108.42
	SQC4 (0.300 μg/mL)	24 h	92.65	89.71	95.69
		48 h	95.27	92.85	97.74
		72 h	100.51	98.34	102.73
Freeze-thaw stability	SQC1 (50.0 μg/mL)	1×	96.28	93.40	99.25
		2×	106.23	105.22	107.25
		3×	108.65	107.66	109.64
	SQC4 (0.300 μg/mL)	1×	98.44	93.83	103.28
		2×	104.60	99.51	109.94
		3×	106.18	103.56	108.86
Long-term stability at approximately −20 °C	SQC1 (50.0 μg/mL)	2 d	107.14	104.84	109.48
		4 d	98.21	97.09	99.34
		3 w	107.58	106.12	109.06
	SQC4 (0.300 μg/mL)	2 d	100.88	94.53	107.66
		4 d	94.68	91.88	97.57
		3 w	105.25	101.88	108.73
Long-term stability at approximately −70 °C	SQC1 (50.0 μg/mL)	2 d	107.78	106.70	108.87
		4 d	96.69	95.83	97.55
		3 w	108.41	106.81	110.04
	SQC4 (0.300 μg/mL)	2 d	106.97	104.91	109.07
		4 d	93.77	90.92	96.71
		3 w	105.79	104.15	107.45

mode. Both methods were robust and reliable, however their run times were approx. 15 min with rather high LLOQ values of 2 μg/mL and 20 μg/mL, respectively. Although LC–MS working in full-scan mode may offer adequate specificity and sensitivity, MS/MS triple quadrupole mass spectrometry used in the selected reaction monitoring mode (SRM) provides surpassing speed, sensitivity and selectivity in quantitative analysis. In comparison to the reported LC–MS methods in full-scan mode, the present LC–MS/MS method is more sensitive and faster because SRM maximizes signal intensity for selected product ions as it increases the duty cycle, *i.e.* the time the quadrupole remains parked on that one ion.

In summary and with respect to the fact that in pharmacokinetic studies naproxen plasma levels will usually spread from 5 to 120 μg/mL [40] comparable methods covering this range have been developed by Miksa et al. [22], Upton et al. [31], Tashtoush and Al-Taani [41] and Phillips and Wellner [53]. However, they either show long run times of 15 or 18 min or lack the use of an internal standard or employ techniques less common such as IACE. These drawbacks leave those methods less suitable for routine analysis of high sample numbers.

Many of the methods reported in Table 3 have been applied in pharmacokinetic studies [26,60–68]. However, besides interferences by co-eluting drugs, especially other NSAIDs, inter-day, intra-day CV's, and recovery data, no additional validation experiments are provided by most of the methods listed in Table 3. Our method provides full information about quality and recovery data of the developed assay, stability of the analyte pre and post sample work up and under different storing conditions, as well as the influence of hemolyzed plasma on the determination of naproxen. The validated assay is in agreement with guidelines from regulatory authorities [14–16] and was performed according to the rules of GLP.

3.4. Clinical study

A clinical study was performed to test the assay's suitability by investigating the pharmacokinetics of naproxen and comparing them to literature reports. As pharmacokinetic measures of systemic exposure C_{max}, t_{max}, and AUC were used for bioavailability evaluation [15], while half live was used to describe its elimination from the body.

The suitability of the method was used in a total of 1226 samples so far. The coefficient of correlation of resulting linear regressions was at least 0.998. The inter-day precision and accuracy of the spiked quality control standards of naproxen in human plasma analyzed with the batches of study samples ranged from 4.4 to 6.7% and from 96.1 to 100.9%, respectively, and were judged acceptable. This excellent performance is most important in studies where PK/PD-relationships (pharmacokinetics, pharmacodynamics or toxicodynamics) have to be established, since the biological measurement in PK/PD-relationships has usually a higher inherent variability that is often difficult to avoid. Fig. 4 shows the mean plasma concentration profile (including the standard deviations) of naproxen after oral administration of a 220 mg formulation tablet to 28 healthy volunteers. The mean peak concentration (C_{max}) of 43.7 μg/mL for naproxen was attained at 1.09 h after administration of the reference product. The mean half-life was 18.5 h.

Table 4 shows the pharmacokinetic parameters of naproxen after administration of a tablet of 220 mg naproxen sodium in comparison to pharmacokinetic parameters reported in previously published studies. Dose-dependent studies of naproxen in healthy volunteers after single and multiple doses observed a non-

Table 3
Reported methods for the quantification of naproxen in clinical samples (grouped by analytical techniques and descending years of publication).

Method[a]	Matrix[b]	Linearity range (μg/mL)	Approx. run time (min)	Sample volume (mL)	Sample preparation[c]	Inter-day CV (%)	Inter-day accuracy (%)	Recovery (%)	Correlation coefficient	Internal standard	Additional validation experiments[d]
LC–MS/MS (current paper)	Plasma	0.1–50	2	0.1	PP	4.4–9.4	94.4–103.1	90	≥0.9980	Ketoprofen	1–4a, 5
Sultan et al. [21] LC–MS	Pharmaceuticals	0.5–15	15	0.5	LLEx	3.98–5.94[e]	n.r.	97.3–99.0	0.9920	n.r.	6
Miksa et al. [22] LC–MS	Bovine serum	0.05–20	15	0.4	PP	≤10	n.r.	>79	>0.9930	n.r.	6
Zakeri-Milani et al. [24] LC-UV	Intestinal perfusate	15.6–250	20	n.a.	Filtration	0.3–1.03	n.r.	n.r.	0.9994	n.r.	n.r.
Aresta et al. [35] LC-UV	Urine	0.2–20	25	0.15	SPME	n.r.	n.r.	90–99	>0.999	n.r.	n.r.
Sultan et al. [21] LC-UV	Blood, plasma, erythrocytes	2.5–15	15	0.5	LLEx	n.r.	n.r.	85.3–94.4	0.9932	Salicylic acid	6
Hirai et al. [36] LC-UV	Urine	0.005–1	20	1	SPE	2.8–8.3	n.r.	92–93	0.99995	Indomethacin	6
Marzo et al. [26] LC-UV	Plasma	1–100	5	0.5	LLEx	≤2.3	n.r.	ca. 100	n.r.	n.r.	n.r.
Lo et al. [37] LC-UV	Blood	25–100	25	1	LLEx	n.r.	n.r.	89–103	0.9962	Heptabarbitone	6
Blagbrough et al. [25] LC-UV	Plasma, synovial fluid	5–100	10	0.5	LLEx	n.r.	n.r.	100	>0.995	Diphenyl-acetic acid	n.r.
Vree et al. [32] LC-UV	Plasma, urine	1.5[f]	>20	0.1	PP	2.3–10.3	n.r.	n.r	n.r.	n.r.	n.r.
Streete [33] LC-UV	Horse serum	52–700	5	0.01	PP	1.6–2.1	101.4–101.5	99.3–100.5	0.9987	Pentobarbital	6
Satterwhite and Boudinot [28] LC-UV	Rat plasma	5–50	12	0.1	LLEx	2.60–2.68	n.r.	100	n.r.	Ketoprofen	n.r.
Levine and Caplan [29] LC-UV	Serum, blood	1–200	5	0.5	LLEx	n.r.	n.r.	n.r.	n.r.	Phenolphthalein	6
van Loenhout et al. [23] LC-UV	Plasma, urine	1–100	15	0.5	LLEx	0.71–2.40	98.9–115.2	95	0.9999	Diflunisal	1
Broquaire et al. [30] LC-UV	Plasma	1–120	8	0.1	LLEx	n.r.	n.r.	94–102	n.r.	(Naphthalene-2-yl)acetic acid	n.r.
Upton et al. [31] LC-UV	Plasma, urine	0.2–100	18	1	LLEx	1.5–2.3	n.r.	80–90	n.r.	Ketoprofen	6
Slattery and Levy [27] LC-UV	Plasma, serum	8–80	12	0.5	LLEx	2.4–5.1	94.7–105.0	96.4 ± 4	n.r.	*p*-Chloro-warfarin	1
Dusci and Hackett [34] LC-UV	Serum	1–40	5	0.1	PP	n.r.	n.r.	88–94	n.r.	n.r.	6
Tashtoush and Al-Taani [41] LC-FLUO	Plasma	0.5–80	5	0.1	PP	n.r.	n.r.	95.4–99.9	≥0.999	n.r.	6
Mikami et al. [42] LC-FLUO	Urine	0.008–0.096	10	0.5	SPE	n.r.	n.r.	92.6–98.1	≥0.999	Methyl *p*-toluate	n.r.
Andersen and Hansen [20] LC-FLUO	Plasma, urine	1–50	8	0.2	PP	n.r.	n.r.	80–93	n.r.	n.r.	n.r.
van Loenhout et al. [23] LC-FLUO	Plasma, urine	0.1–10	15	0.5	LLEx	1.2–3.9	96.8–101.4	95	0.9997	2-(6-Ethoxy-naphthalen-2-yl)propanoic acid	1
Shimek et al. [38] LC-FLUO	Plasma	2.5–70	>30	0.1	LLEx	n.r.	n.r.	66.6	0.9913	n.r.	1, 6
Burgoyne et al. [39] LC-FLUO	Serum	1–100	10	0.1	LLEx	n.r.	n.r.	72–84	>0.95	n.r.	n.r.

Table 3 (*Continued*)

Method[a]	Matrix[b]	Linearity range (μg/mL)	Approx. run time (min)	Sample volume (mL)	Sample preparation[c]	Inter-day CV (%)	Inter-day accuracy (%)	Recovery (%)	Correlation coefficient	Internal standard	Additional validation experiments[d]
Westerlund et al. [40] LC-FLUO	Plasma, urine	2–30	15	0.5	LLEx	n.r.	n.r.	93–107	n.r.	(6-Methoxy-naphthalene-2-yl)acetic acid	n.r.
Larsen and Marinelli [43] GC–MS	Plasma	0.005[f]	n.r.	1.0	LLEx	<10[g]	<110[g]	n.r.	n.r.	d_3-Naproxen	n.r.
Wan and Matin [44] GC–MS	Urine	1–40	20	0.5	LLEx	n.r.	n.r.	n.r.	n.r.	2-(6-Propoxy-naphthalen-2-yl)propanoic acid	n.r.
Weber et al. [45] GC-FID	Serum	0.6–150	10	1	LLEx	n.r.	n.r.	n.r.	n.r.	Methyl (6-methoxynaphthalene-2-yl)acetate	n.r.
Desager et al. [46] GC-FID	Plasma	5–120	6	1	LLEx	n.r.	n.r.	93–99	n.r.	(6-Methoxy-naphthalene-2-yl)acetic acid	n.r.
Mortensen et al. [47] FLUO	Serum	0.01–5	n.r.	1	LLEx	7–10	n.r	64	0.999	n.a.	6
Held [48] FLUO	Serum, urine	25–100	n.r.	0.1	LLEx	n.r.	n.r.	71.1	n.r.	n.a.	n.r.
Anttila [49] FLUO	Plasma, serum	5–200	n.r.	0.1	LLEx	n.r.	n.r.	97–101	n.r.	n.a.	n.r.
Guermouche et al. [50] HPTLC-UV	Rat serum	2–100	n.r.	0.1	LLEx	n.r.	n.r.	92–96	0.992	Benzophenone	6
Abdel-Moety et al. [51] TLC-UV	Urine	≤3	4	1	LLEx	n.r.	n.r.	93.9	n.r.	n.a.	n.r.
Knie [52] HPTLC-FLUO	Serum	5–80	8	0.1	PP	n.r.	n.r.	93.0–99.6	≥0.999	n.a.	n.r.
Phillips and Wellner [53] IACE	Plasma	0.1–100	5	n.r.	Ultrafiltration	5.81	n.r	n.r.	n.r.	n.r.	n.r.
Albrecht and Thormann [54] MECC-LIF	Tissue	0.1–2	6	0.1	LLEx	n.r.	n.r.	80	n.r.	Sodium salicylate	n.r.
Sun et al. [55] LC-ECL	Urine	0.04–2	6	1	Centrifugation	4.9–6.1	n.r.	95–101	0.993	n.a.	n.r.
Kazemifard and Moore [56] LC-EC	Plasma	0.02–10	15	0.02–0.2	SPE	n.r.	n.r.	n.r.	>0.999	Indomethacin	6
Pérez-Riuz et al. [57] MS-RTP	Serum, urine	0.09–4.5	10	0.1	Centrifugation	n.r.	n.r.	96–101	0.999	n.a.	6

n.a.: not applicable, n.r.: not reported.

[a] MS-RTP: micelle stabilized room temperature phosphorescence, LC-EC: liquid chromatography with electrochemical detection, LC-ECL: liquid chromatography with electrogenerated chemiluminescence detection, MECC-LIF: micellar electrokinetic capillary chromatography with laser-induced fluorescence detection, IACE: immunoaffinity capillary electrophoresis.

[b] Human samples if not stated otherwise.

[c] PP: protein precipitation, LLEx: liquid–liquid extraction, SPE: solid-phase extraction, SPME: solid-phase microextraction.

[d] 1: specificity, 2: stability (short-term (a), long-term (b), post-preparative (c), freeze–thaw (d), room temperature (e)), 3: dilution, 4: influence of hemolyzed (a), lipemic (b) plasma, 5: influence of different individuals, 6: interference of drugs.

[e] Intra-day precision.

[f] LLOQ.

[g] For 20 ng/mL.

Table 4
Literature values for naproxen pharmacokinetic parameters (mean ± standard deviation) in healthy subjects after overnight fasting and administration of a conventional tablet under fasting conditions.

Reference	Number of subjects (gender[a])	Last time point (h)	Dosis/formulation (mg/tablet)	$AUC_{0\rightarrow last}$ (μg × h/mL)	$AUC_{0\rightarrow\infty}$ (μg × h/mL)	C_{max} (μg/mL)	t_{max} (h)	$t_{1/2}$ (h)
Current paper	28 (13f, 15m)	72	200	585 ± 80.93	626 ± 101.7	43.7 ± 7.3	1.09 ± 0.77	18.54 ± 3.34
Anttila et al. [61]	8 (4f, 4m)	72	250	n.r.	797 ± 191	n.r.	n.r.	17.7 ± 3.0
Caillé et al. [62]	6 (6m)	48	500	n.r.	1624 ± 99	95.6 ± 5.8	2.2 ± 0.5	15.9 ± 0.6
Caillé et al. [63]	12 (n.r.)	48	500	n.r.	1310.4 ± 79.4	82.7 ± 3.4	1.4 ± 0.2	15.5 ± 1.0
Charles et al. [72]	14 (1f, 13m)	60	500	n.r.	1211	71.4	1.5	n.r.
Ling et al. [64]	6 (6m)	48	750	n.r.	1435 (15)[b]	93.2 (7)[b]	1.7 (31)[b]	17.2 (11)[b]
Marzo et al. [26]	12 (6f, 6m)	24	502	710.3	1024.0	63.8	1.1	14.1
Niazi et al. [60]	28 (n.r.)	72	250	n.r.	560.5 ± 29.7	35.48 ± 1.54	2.86 ± 0.32	12.27 ± 0.64
Niazi et al. [60]	28 (n.r.)	72	500	n.r.	942.24 ± 41.64	64.05 ± 2.12	2.25 ± 0.33	13.34 ± 1.29
Ryley et al. [65]	12 (12m)	60	750 (Canadian tablet formulation)	1393.4 ± 346.2	1488.4 ± 386.6	97.3 ± 26.5	2.4 ± 1.2	16.1
Ryley et al. [65]	12 (12m)	60	750 (US tablet formulation)	1391.1 ± 312.4	1491.3 ± 342.7	98.6 ± 25.2	2.3 ± 1.1	16.5
Strocchi et al. [66]	12 (12m)	48	750	n.r.	1547 ± 235	88.9 ± 11.9	1.8 ± 1.0	17.8 ± 2.64
Vree et al. [67]	9 (5f, 4m)	120	500	n.r.	1140 ± 171	62.2 ± 11.1	1.5 ± 0.68	24.7 ± 6.4
Zhou et al. [68]	10 (10m)	48	500	1206.6 ± 121.7	1427.8 ± 192.8	87.3 ± 15.5	2.6 ± 1.5	17.7 ± 3.0

n.r.: not reported.
[a] m: male, f: female.
[b] Mean (% CV).

Fig. 4. Mean plasma profile of naproxen concentration *vs.* time following a 220 mg oral dose of a naproxen sodium tablet to healthy volunteers ($n = 28$).

linear relationship between naproxen and plasma concentrations at higher doses [69,70]. Segre [71] reported a linear relationship between naproxen dose and plasma concentrations within 100–300 mg single dose, and a non-linear relationship at multiple doses of 375–750 mg naproxen. However, Niazi et al. [60] observed a non-linear relationship between naproxen dose and plasma concentrations at 250 and 500 mg single dose naproxen.

The non-linearity in the reported studies is expressed in a less than proportional increase of the AUC and C_{max} when compared to the dose. The plasma protein binding sites are assumed to be saturated at high naproxen doses, which results in a higher concentration of unbound naproxen and leads to a higher excretion rate and clearance [60]. This unbound naproxen concentration was shown to be proportional to naproxen concentrations at 500, 1000, and 1500 mg doses [69]. For this reason the PK parameters in Table 4 are not shown dose-corrected.

The reported mean t_{max} and $t_{1/2}$ values in Table 4 range from 1.09 to 2.86 h and from 12.27 to 24.7 h, respectively. Administration of a naproxen sodium formulation leads to a shorter t_{max} due to the better solubility than the free acid [5,26]. As can be seen, the determined t_{max} and $t_{1/2}$ values of our study are comparable to the values of the other studies, while the AUC and the C_{max} values in general show a greater variability between different reports, especially at higher doses. At comparable doses, *e.g.* 200 or 250 mg, our AUC-values are in good agreement with those of other reports. Additionally to the non-proportional dose to AUC relationship, an inter-study variability may be observed due to non-specific effects like dosage accuracy, adherence to dietary restrictions and drug assay accuracy. In the present study, we minimized non-specific variability for pharmacokinetic parameters by using strict adherence to dietary restrictions and the development of a highly accurate drug assay.

4. Conclusion

The developed and validated LC–MS/MS assay for naproxen in human plasma is simple, fast, reliable, sensitive, precise and accurate. The method employs an automated acetonitrile protein precipitation, which reduces the preparation time and allows quantification of naproxen in human plasma for concentrations ranging from 0.100 to 50.0 μg/mL using 100 μL of plasma. The major advantage of the LC–MS/MS described here is the simultaneous achievement of high absolute recovery (90.0%), acceptable sensitivity (LLOQ = 0.100 μg/mL), high inter-day precision (≤9.4%), high accuracy (98.8%) of the LLOQ, and excellent linearity ($r^2 \geq 0.998$)

combined with a short run time of only 2 min. Up to 650 samples can be run on one LC–MS/MS machine per day. These characteristics make the method suitable not only for monitoring naproxen plasma levels in patients, but also for the precise and accurate measurement of low concentrations of naproxen. This is necessary, both, in the context of pharmacokinetic studies after single dose and when crucial PK/PD-relationships have to be established to assess the compound's beneficial or detrimental effects. In view of the ongoing discussion on adverse effects of NSAIDs it is important that bioanalytical methods validated according to today's standards must be available to investigate the relationship between plasma levels and such effects. The low intra-individual variability (ANOVA-CV) in the values of C_{max} and $AUC_{0\rightarrow t}$ (8.61 and 5.69%, respectively) documents that this bioanalytical method, as well as an appropriate study design eliminated most sources of non-specific variation.

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