Differentiation between naproxen, naproxen–protein conjugates, and naproxen–lysine in plasma via micellar electrokinetic capillary chromatography—a new approach in the bioanalysis of drug targeting preparations

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Pharmacotherapy through the targeting of drugs is a promising new approach that requires adequate analytical methods capable of differentiating between the free drug, the drug carrier, and metabolites. Using micellar electrokinetic capillary chromatography (MECC), we report the separation of naproxen (NAP) from NAP covalently coupled to human serum albumin or to mannosylated serum albumin and the metabolite naproxen–lysine. An assay for selective analysis of the different forms of NAP by direct plasma injection was developed with salicylate as internal standard and solute detection by laser-induced fluorescence. Compared with previously applied techniques, including HPLC and total plasma fluorescence, MECC offers the advantage that free and covalently bound NAP can be differentiated in one run and can be accurately monitored in microliter quantities of plasma. Summation of all NAP equivalents determined by MECC revealed data that compare well with those produced by total plasma fluorescence and HPLC.

Drug targeting is a promising approach in modern pharmacotherapy. Cell-specific delivery of antitumor agents, antivirals, and also antiinflammatory drugs have been described [1]. Naproxen (NAP), (S)-6-methoxy-α-methyl-2-naphthaleneacetic acid (for structure see Fig. 1A), is a widely used nonsteroidal antiinflammatory drug.3 Its antipyretic and analgesic effects are related to the inhibition of cyclooxygenase, a major enzyme in the arachidonic acid conversion pathway, resulting in a decrease of prostaglandin formation. Coupling NAP to human serum albumin (HSA) or mannosylated HSA (Fig. 1B) as carriers provides a means for specific delivery of this compound to endothelial and Kupffer cells of the liver [2]. Interestingly, this biochemical transformation profoundly alters not only the pharmacokinetic behavior and cellular distribution of the parent drug [1], but also has a marked influence on its pharmacological effects in acute [3] and chronic [4] liver disease. With regard to in vitro and in vivo results demonstrating successful targeting of NAP to the liver, growing interest emerges concerning the pharmacokinetic properties of the different NAP conjugates and their metabolites in biological fluids and organs. For this purpose, the simultaneous determination of free NAP, protein conjugates of NAP, and the primary metabolite naproxen–lysine (NAPLYS) is of high interest. No method has yet met this challenge.

For the determination of NAP, its metabolites, and enantiomers in plasma, synovial fluid, and urine, various methods based on HPLC have been developed [5–11]. Furthermore, capillary electrophoretic techniques have been applied to the determination of NAP in human serum and plasma [12–15]. In a previous investigation from our laboratory, the possibility of monitoring total plasma concentrations of NAP by using micellar electrokinetic capillary chromatography (MECC) with direct plasma injection has been demonstrated [15]. In MECC,
surfactants [e.g., sodium dodecyl sulfate (SDS)] above their critical micelle concentrations are added to the running buffer, permitting the separation of uncharged solutes on the basis of differential partitioning. For charged components, separation is governed by electrophoresis, partitioning between the two phases, and electrostatic interactions and solute complexation with the surfactant. One interesting and appealing feature of MECC with dodecyl sulfate micelles is the possibility of directly injecting a tiny (few nanoliters) amount of a proteinaceous body fluid (e.g., plasma) onto an uncoated fused-silica capillary at one end and detecting the separated compounds as they pass an on-column absorbance or fluorescence detector placed towards the other capillary end [13–17].

Previously, plasma concentrations of NAP-HSA and of NAP conjugated to mannosylated HSA (NAP-HSA-MAN) have been determined as total plasma fluorescence with excitation and emission wavelengths of 330 and 360 nm, respectively [18]. Although this is a rapid and simple approach, it does not differentiate between the protein conjugate, free NAP originating from the drug formula-
tion (up to ~20 g/kg), and the metabolite NAPLYS. By using HPLC, free NAP and NAPLYS were determined by analyzing plasma extracts and extracts of hydrolyzed plasma [2, 19]. Through subtraction, indirect information on the plasma concentration of the conjugate was obtained. MECC with its capability of handling low- and high-molecular-mass compounds appears to be the ideal analytical free solution approach for the simultaneous determination of carriers and metabolites used in drug targeting. In this paper, analysis of different forms of NAP-containing drugs in rat plasma by using MECC with direct sample injection (MECC-DSI) is discussed, and data obtained are compared with those produced by HPLC and a fluorometric assay. MECC-DSI with on-column laser-induced fluorescence (LIF) detection of solutes is shown to be a selective, simple, and economical approach for the assessment of the distribution and metabolism of NAP–protein conjugates.

**Materials and Methods**

**Drugs, chemicals, animal experiments, and plasma samples.** All chemicals used were of analytical or research grade. SDS was purchased from BHD Laboratory Supplies. Na₂HPO₄, Na₂B₄O₇, and sodium salicylate were obtained from Merck. Synthesis of NAP-HSA and NAP-HSA-MAN was performed according to Franssen et al. [18]. NAPLYS was synthesized as described by Grolleman et al. [20] and Franssen et al. [18]. The batch of NAP-HSA used in the present study contained 23 molecules of NAP per molecule of HSA, whereas the NAP-HSA-MAN batch consisted of 9 mol of NAP and 10 mol of MAN bound per mole of HSA (determined by analysis of drug and protein content [21, 22]). Both preparations contained some unreacted free NAP. NAP–protein conjugates were freshly dissolved in saline and administrated to male Wistar rats (250–350 g, anesthetized with pentobarbital 50 mg/kg) via intravenous bolus injection. Plasma samples were withdrawn over a period of 3 h thereafter. Animal experiments were performed at the Department of Pharmacokinetics and Drug Delivery, Groningen, The Netherlands. Plasma samples were stored at −18 °C until analysis.

**Electrophoretic instrumentation and running conditions for MECC.** MECC was performed on a P/ACE 5510 capillary electrophoresis system (Beckman Instruments) featuring automated capillary rinsing, sampling, temperature control of the capillary, data collection, storage, and evaluation. Fused-silica capillaries (Polymicro Technologies) of 50-μm i.d. were used. The effective capillary length was 20 cm (total length of 27 cm). A constant voltage of 8 kV was applied (current ~30 μA) and the anode was on the sampling side. Sample injection was effected by applying positive pressure at 3448 Pa (0.5 psi) for 1 s. The capillary temperature was maintained at 20 °C. The sample carousel was at ambient temperature. Solute detection was effected by LIF with an air-cooled 10 mV HeCd laser (Liconix), which emits at 325 nm. A 366-nm emission filter...
was used. The photomultiplier tube gain was set to 1. Data were evaluated with the Gold Software package version 8.1 (Beckman). Capillaries were conditioned between runs by application of positive pressure [34.48 kPa (5 psi)] with 0.1 mol/L NaOH (3 min), water (3 min), and running buffer (3 min). The running buffer consisted of 10 mmol/L sodium tetraborate, 6 mmol/L disodium hydrogen phosphate, and 75 mmol/L SDS (pH ~9.2). The buffer vials were replenished every 5–6 runs.

**Calibrator solutions, preparation of samples, and principle of quantification by MECC.** Aqueous calibrator solutions of NAP [10 mg/L (43.4 µmol/L)], NAP-HSA [1.5 g/L (20.9 µmol/L)], and NAP-HSA-MAN [3.0 g/L (41.7 µmol/L)] as well as a methanolic solution of NAPLYS [0.264 g/L (737.4 µmol/L)] were prepared and diluted with a stock solution of 16.6 g/L (100 mmol/L) sodium salicylate, which was used as internal standard (IST). Aliquots of 50 µL of plasma and IST solution were pipetted into a 600-µL Eppendorf plastic vial and vortex-mixed for ~2 s. Thereafter, the plastic vial was cut down to half size and inserted into the vial holder of the P/ACE 5510. Quantification was based upon internal, multilevel calibration by using the peak area ratio of the compound to the IST.

**HPLC assay.** For the determination of total NAP concentrations, the method of Franssen et al. [19] was used with some minor modifications. Briefly, plasma samples (50 µL) were first subjected to alkaline hydrolysis for 72 h at 80 °C by using 1 mL of 5 mol/L NaOH. Then, the samples were acidified with 5 mol/L HCl to pH 1.5, 100 µL of IST solution [flurbiprofen, 10 mg/L (40.9 µmol/L)] was added, and NAP was extracted with 6 mL of dichloromethane. After evaporation of the organic phase under a steady stream of nitrogen, the residue was redissolved in 300 µL of mobile phase and 100 µL was injected into the HPLC column. The mobile phase consisted of water: acetonitrile:acetic acid (60:40:1 by vol) and the flow rate was 1.5 mL/min. Separation was done on a reversed-phase C18 column (Nucleosil C18 ET 250/8/4, Macherey Nagel). NAP was detected by fluorometry at excitation and emission wavelengths of 334 and 360 nm, respectively (Perkin-Elmer Fluorescence Spectrophotometer 204). The IST was monitored simultaneously by UV detection at 254 nm with a Spectroflow 773 (Kratos Analytical) and a D-2000 Chromato-Integrator (Merck-Hitachi). Retention times for NAP and IST were determined to be 8.2 and 14.6 min, respectively: The limit of detection (LOD) for NAP was found to be 100 µg/L. Calibration graphs for NAP [1.25–25.0 mg/L (5.4–108.5 µmol/L)] were constructed by adding calibration solutions to whole heparinized rat plasma.

**Plasma fluorescence assay.** Plasma concentrations of NAP–protein conjugates were also estimated via total plasma fluorescence according to Franssen et al. [18] on a fluorescence spectrophotometer Aminco SLM SPF 500 (SLM Instruments) by using excitation and emission wavelengths of 330 nm and 360 nm, respectively. Plasma samples (20 µL) were diluted with Krebs buffer (118 mmol/L NaCl, 5.0 mmol/L KCl, 1.1 mmol/L MgSO4 \(\cdot\) 7 H2O, 2.5 mmol/L CaCl2 \(\cdot\) 2 H2O, 1.2 mmol/L KH2PO4) to a final volume of 2 mL and vortex-mixed for 5 s. Calibration graphs for NAP-HSA [100–1500 mg/L (1.4–20.9 µmol/L)] and NAP-HSA-MAN [250–3000 mg/L (3.5–41.7 µmol/L)] were constructed by adding calibrator solutions to rat blood plasma.

**Results and Discussion**

Data obtained with plasma samples containing NAP-HSA and salicylate as IST are depicted in Fig. 2. For the blank rat plasma (panel A), no peak was detected. A typical electropherogram containing blank rat plasma supplemented with NAP-HSA, NAPLYS, and IST is depicted in panel B. The NAP detected stems from the NAP-HSA calibrator. Panel C shows an electropherogram of a plasma sample drawn 120 min after intravenous administration of 22 mg/kg NAP-HSA to an anesthetized rat. In addition to NAP-HSA and free NAP, NAPLYS (metabolite of NAP-HSA) could be clearly detected. The low-molecular-mass substances are shown to form sharp peaks, whereas NAP-HSA is registered as broader peak (see below). All analytes are completely separated, revealing retention times of 4.4, 5.4, 8.1, and 9.4 min for NAP, IST, NAP-HSA, and NAPLYS, respectively. Corresponding data with plasma samples containing the mannosylated conjugate NAP-HSA-MAN are presented in Fig. 3. Panels B and C depict data obtained with a calibrator plasma sample and with rat plasma drawn 180 min after intravenous injection of 48 mg/kg NAP-HSA-MAN, respectively. As with NAP-HSA, NAP-HSA-MAN appears as a broad peak. It is important to note that LIF solute detection as used here selectively visualizes the components of interest only. Endogenous substances are not detected. This is similar to the conditions used for MECC-based immunochemical drug assays [16].

In MECC-DSI, proteins are solubilized by dodecyl sulfate and elute as broad peaks [14, 15]. NAP and NAPLYS elute in front and after the rat plasma proteins, respectively, thereby producing sharp peaks. The protein conjugates, however, do not completely separate from other proteins and therefore appear as broad peaks. However, as illustrated with the data presented in Fig. 4, the two NAP–protein conjugates could be separated in the presence of the plasma proteins. NAP-HSA-MAN eluted in front of NAP-HSA. Interestingly, application of the NAP-HSA-MAN calibrator dissolved in water produced a rather sharp peak (see left inset in Fig. 4), whereas a relatively broad peak was observed for NAP-HSA that was sampled in water (right inset in Fig. 4). This is likely to be due to the difference and possible variation in drug loading of the carriers: HSA contains 23 molecules of the hydrophobic NAP per protein molecule in the case of
Fig. 2. Electropherograms obtained with direct plasma injection with (A) rat plasma blank; (B) rat plasma supplemented with 70 mg/L (977.6 nmol/L) NAP-HSA, 3.82 mg/L (10.7 μmol/L) NAPLYS, and 44.4 mg/L (0.28 mmol/L) IST; and (C) rat plasma withdrawn 120 min after injection of 22 mg/kg NAP-HSA.

Conditions are as described in Materials and Methods.

Fig. 3. Electropherograms obtained with direct plasma injection with (A) rat plasma blank; (B) rat plasma supplemented with 500 mg/L (6.95 μmol/L) NAP-HSA-MAN, 4.17 mg/L (11.6 μmol/L) NAPLYS, and 26.7 mg/L (0.17 mmol/L) IST; and (C) rat plasma withdrawn 180 min after injection of 48 mg/kg NAP-HSA-MAN.

Conditions are as described in Materials and Methods.
Quantification of NAP and its derivatives was based upon internal, four to six-level calibration by using the peak area ratio of the compound to the IST and having 0.5–25 mg/L (2.17–108.6 μmol/L), 25–600 mg/L (0.35–8.38 μmol/L), 25–1000 mg/L (0.35–13.9 μmol/L), and 3–60 mg/L (8.38–167.6 μmol/L) concentration ranges for NAP, NAP-HSA, NAP-HSA-MAN, and NAPLYS, respectively. Calibration graphs were linear, with \( F \)-values for all compounds >200 (\( P < 0.0001 \)). The \( y \)-intercepts were significantly smaller than the smallest calibrator values and were thus negligible. For all compounds, intra- and interday CVs were <10% (\( n \geq 5 \)). Table 1 summarizes the analytical characteristics of the assay.

Table 1. Characteristics of the MECC assay with direct injection of rat plasma

<table>
<thead>
<tr>
<th>Compound</th>
<th>LOD, nmol/L</th>
<th>CV, %</th>
<th>Intraday</th>
<th>n</th>
<th>Interday</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAP</td>
<td>140</td>
<td>1.92</td>
<td>7</td>
<td>4.83</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>NAP-HSA</td>
<td>7</td>
<td>1.43</td>
<td>7</td>
<td>7.68</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>NAP-HSA-MAN</td>
<td>140</td>
<td>8.36</td>
<td>7</td>
<td>4.67</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>NAPLYS</td>
<td>70</td>
<td>0.63</td>
<td>6</td>
<td>1.90</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

LOD is based on a signal/noise ratio = 3.

Fig. 5. Elimination curves of NAP and NAP–protein conjugates [(A) NAP-HSA and (B) NAP-HSA-MAN] in anesthetized rats during 180 min after drug administration together with the temporal increase of the primary metabolite NAPLYS.

Conditions are the same as those of Figs. 2C and 3C, respectively.
With MECC-DSI it was possible to assess the elimination of free and conjugated NAP and to register the appearance and temporal increase of the metabolite NAPLYS (Fig. 5). It is important to note that with this assay, total plasma concentrations of NAP, i.e., free NAP together with NAP noncovalently bound to plasma proteins, are determined. The proteinaceous material can be applied without any sample pretreatment, i.e., no time-consuming sample cleanup (e.g., extraction, derivatization) before analysis is necessary. As the separation of the analytes by MECC-DSI can be performed in one single run, the load of samples and consumption of organic solvents is minimized. Furthermore, automation and run times of ≤10 min make this method effective and attractive for the determination of NAP, NAP–protein conjugates, and their primary metabolite NAPLYS.

To demonstrate the efficacy of the electrokinetic assay, MECC and HPLC data of 61 plasma samples were assessed. The data presented in Fig. 6A represent total NAP concentrations determined after hydrolysis and extraction with HPLC. These values were compared with the total NAP equivalents calculated from the various drug concentrations that were determined by MECC-DSI. Linear regression analysis of the data pairs (Fig. 6A) revealed a correlation coefficient, y-intercept, and slope of 0.860, 9.49 μmol/L, and 0.910, respectively. These data indicate a good linear relation. Furthermore, plotting the difference against the mean of the corresponding data pairs according to Bland and Altman provided a better insight into the equality of the two sets of data (Fig. 6B). In relation to the calibration range, the mean difference between MECC and HPLC data (–3.95 μmol/L) was small and the data appear to be evenly distributed, indicating that the two methods provide comparative total plasma concentrations of NAP. Fifty eight of the 61 data points are within the region defined by the mean difference ± 2 SD (region bracketed by broken lines). It is important to realize that with HPLC the concentrations of NAP conjugates can be determined only indirectly, i.e., via hydrolysis of the NAP–lysine bond. The possibility of distinguishing between all NAP-containing compounds with the MECC assay therefore is a clear advantage.

Comparative data obtained by the fluorometric assay and MECC-DSI of 50 plasma samples are presented in Fig. 7A. Linear regression analysis revealed a linear relation (r = 0.884) with a calculated intercept of –0.155 μmol/L and a slope close to one (0.958). These results indicate that both methods provide comparable drug concentrations. This is further underlined in Fig. 6B, in which the difference against the mean of the corresponding data pairs is plotted. The mean difference between the MECC and fluorometric assay data was 0.306 μmol/L. Only one of 50 data points was outside the region defined by the mean difference ± 2 SD. The fluorometric assay is based on the measurement of total plasma fluorescence, and the data are not corrected via use of an IST. The different NAP compounds are not distinguished. Drug concentrations assessed with total fluorometric assay can only be used as a first estimate to characterize plasma clearance of the conjugate in the initial (30 min) period after injection, in which metabolism of the conjugate plays only a minor role because of a lag time in the lysosomal degradation of the HSA conjugate. After this period they not only include contributions from small amounts of free NAP, which is coadministered with NAP-HSA and NAP-HSA-MAN (typically <20 g/kg), but also from the metabolite NAPLYS. As is shown with the data presented in Fig. 5, concentrations of free NAP and NAPLYS are not constant: The concentrations of NAP are slowly decreasing with time, whereas those of NAPLYS could only be detected 20
In conclusion, MECC with direct plasma injection is shown to provide a selective, simple, rapid, and attractive approach for the simultaneous assessment of newly developed NAP–protein drugs used in drug targeting, their primary metabolite NAPLYS, and free NAP. No expensive column and no sample preparation are required to separate the different NAP compounds in a single run. The direct application of proteinaceous material and the high degree of automation combined with short run times of maximal 10 min make this MECC method highly effective and economic. The MECC-DSI method is superior to the total plasma fluorescence method, which lacks specificity, and to HPLC, which requires time-consuming sample preparation and more than one run per sample for the estimation of the plasma concentration of a conjugate. Finally, the fact that only microliter quantities of plasma are required for MECC-DSI is an important advantage for kinetic investigations in small laboratory animals. Although this paper reports data obtained with rat plasma, the same assay could be used for monitoring the NAP compounds in human plasma [24]. These promising drug targeting preparations are not yet used in human studies; however, they represent an exciting new approach in pharmacotherapy.

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