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Determination of naproxen in liver and kidney tissues by electrokinetic capillary chromatography with laser-induced fluorescence detection

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Abstract

Pharmacotherapy through the targeting of drugs is a promising new approach that requires adequate analytical methods capable of monitoring the free drug, the drug carrier and metabolites in body fluids and organs. A micellar electrokinetic capillary chromatography (MECC) based assay for analysis of naproxen (NAP) in extracts of hydrolyzed liver and kidney tissue homogenates using salicylate as internal standard and solute detection by laser-induced fluorescence is reported. The assay described uses 100 μl of hydrolyzed tissue homogenate and has a detection limit of 0.07 μg/ml. It is shown to be selective, reproducible (at a NAP level of 0.25 μg/ml, intra-day and inter-day R.S.D. values are 3.73% and 6.39%, respectively), simple and economical (operates with inexpensive separation columns and small amounts of chemicals). It has been successfully applied to the assessment of the total NAP content within liver and kidney tissues of male Sprague Dawley rats that have been treated with NAP conjugated to human serum albumin (the drug targeting carrier) and free NAP. Compared to previously applied techniques, including high-performance liquid chromatography, MECC offers the advantage of having lower running costs and lower consumption of organic solvents. © 1998 Elsevier Science B.V.

Keywords: Drug targeting; Naproxen; Anti-inflammatory drugs

1. Introduction

During the past decade, instrumentation for electrokinetic separations in fused-silica capillaries of very small I.D. became available and the feasibility of employing capillary electrophoresis (CE) for drug monitoring in body fluids, including plasma, serum, saliva and urine, has been tested extensively in various laboratories (for recent reviews see [1,2]). Measuring drug levels in tissues is important for the assessment of drug distribution in the body and investigation of past exposure to drugs. There are very few papers reporting the use of CE for the monitoring of a substance in a tissue. Examples described include various endogenous compounds [3–5] and drugs [6–9]. Most prominent is the CE analysis of cocaine and morphine in human hair samples reported by Tagliaro et al. [6,7]. Furthermore, the CE monitoring of the anticancer drug prospidin in human papilloma species [8] and the capillary gel electrophoretic determination of an antisense 20mer phosphorothioate oligonucleotide and some of its metabolites in nude mouse kidney [9] have been described.

Drug targeting is a promising approach in modern pharmacotherapy. It aims at delivering a drug at increased concentrations to its intended site of action while minimizing drug exposure to other organs in

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order to reduce toxicity. Different approaches to achieve this goal have been recently reviewed by Meijer and Molema [10]. The two most common principles are the design of prodrugs which are activated specifically at their intended site of action or the use of carriers, e.g. neoproteins which are taken up by specific receptors on different cell types. The latter concept is exemplified by the drug used in the present study, naproxen (NAP) covalently bound to human serum albumin (HSA).

NAP is a widely used nonsteroidal anti-inflammatory drug. 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 covalently to HSA as a carrier provides a means for specific delivery of this compound to the liver [11–13]. With regard to in vitro and in vivo results, growing interest emerges concerning the pharmacokinetic properties of the different NAP conjugates and their metabolites in biological fluids and organs. Recently, CE techniques have been applied to the determination of NAP in human serum and plasma [14–16]. In a previous investigation from our laboratory, the possibility of simultaneous monitoring of plasma levels of NAP, NAP–protein conjugates and the metabolite NAP–lysine (NAPLYS) employing micellar electrokinetic capillary chromatography (MECC) with direct plasma injection has been demonstrated [17].

Having shown that MECC is the ideal analytical free solution approach for the simultaneous determination of carriers and metabolites employed in drug targeting, investigations leading to the MECC monitoring of these compounds in tissues have commenced. In this paper, analysis of NAP in extracts prepared from hydrolyzed rat liver and kidney tissue homogenates using MECC and laser-induced fluorescence (LIF) detection is discussed. The assay described is shown to be selective, simple, economical and applicable to the assessment of the total NAP content in tissues of male Sprague Dawley (SD) rats after i.v. bolus injection of either the drug targeting compound composed of 23 NAP molecules covalently bound to HSA (NAP_{23}–HSA) or free NAP.

2. Experimental

2.1. Drugs, chemicals, animal experiments and preparation of organ homogenates

All chemicals used were of analytical or research grade. Sodium dodecyl sulfate (SDS) was purchased from BDH (Poole, UK). Na_2HPO_4, $Na_2B_4O_7$ and sodium salicylate were obtained from Merck (Darmstadt, Germany). NAP_{23}–HSA was the same as employed previously [17]. NAP and NAP_{23}–HSA were freshly dissolved in saline and administered to male SD rats (250–350 g, anaesthetized with pentobarbital 50 mg/kg) via i.v. bolus injection. The liver and the kidneys of the rats were removed 15 min after injection of 22 mg NAP_{23}–HSA per kg body mass or 1.5 mg NAP per kg body mass. The animal experiments had been approved by the State's committee for animal experimentation and were performed according to strict federal and international guidelines on animal experimentation. Immediately after organ removal, 1 g of organ tissue (liver or kidney) was homogenized with 10 ml (liver) or 5 ml (kidney) 0.1 M KH_2PO_4 buffer (pH 7.4) using a Polytron PT 3000 homogenizer (Kinematika, Littau, Switzerland) at 30 000 rpm for 30 s. The homogenates were stored at $-18°C$ until analysis.

2.2. Electrophoretic instrumentation and running conditions for MECC

MECC was performed on a P/ACE 5510 capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA) featuring automated capillary rinsing, sampling, temperature control of the capillary, data collection, storage and evaluation. Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm I.D. were employed. The effective capillary length was 20 cm (total length of 27 cm). A constant voltage of 8 kV was applied (current about 30 μA) and the anode was on the sampling side. Sample injection was effected by applying positive pressure at 0.5 p.s.i. for 1 s (1 p.s.i.=6894.76 Pa). The capillary temperature was maintained at 20°C. The sample carousel was at ambient temperature. Solute detection was effected by LIF using an air-cooled 10 mV He–Cd laser

(Liconix, Santa Clara, CA, USA) which emits at 325 nm and a 366 nm emission filter. The photomultiplier tube (PMT) gain was set to 1. Data were evaluated using the Gold Software package version 8.1 or the P/ACE Station Software (Beckman). Capillaries were conditioned between runs by application of positive pressure (5 p.s.i.) employing 0.1 M NaOH (3 min), water (3 min) and running buffer (3 min). The running buffer consisted of 10 mM sodium tetraborate, 6 mM disodium hydrogenphosphate and 75 mM SDS (pH ~9.2). The buffer vials were replenished every 5–6 runs.

2.3. Standard solutions, preparation of calibrators and principle of quantitation

Aqueous standard solutions of NAP [100 μg/ml (434 μM)] and of sodium salicylate [16.6 μg/ml (about 0.1 mM)] were prepared. For preparation of calibrators and controls, aliquots of the NAP solution were added to homogenized organ tissue of untreated rats (4–5 NAP concentration levels between 0.1 and 2.0 μg/ml, corresponding to 1.1–22 μg NAP per gram liver and 0.6–12 μg NAP per gram kidney). Quantitation was based upon internal, multi-level calibration using the peak height ratio of NAP to the internal standard (I.S.).

2.4. Sample preparation

A 100-μl volume of thawed tissue homogenate or 100 μl of calibrator homogenate were incubated with 1 ml of 5 M NaOH at 80°C for 72 h. After hydrolysis, the mixture was acidified with 1.5 ml 5 M HCl and supplemented with 100 μl of I.S. solution (corresponding to an I.S. concentration of 16.6 μg per ml homogenate) and 6 ml dichloromethane. For extraction, the sample was vigorously shaken for 15 min and centrifuged for 10 min at about 1500 g. After removal of the water (upper) phase, the organic phase was evaporated to dryness under a steady stream of nitrogen (~30 min at 37°C) and the residue was redissolved in 100–120 μl of MECC running buffer. Recovery of NAP was determined to be about 80%.

3. Results and discussion

3.1. MECC analysis of NAP in extracts of hydrolyzed tissue homogenates

Data obtained with tissue samples are depicted in Figs. 1–4. For the blank rat liver extract (panel A of Fig. 1) no major peak in addition to that obtained for the I.S. (salicylate) was detected. A typical electropherogram obtained with blank liver homogenate that was spiked with 2 μg/ml of NAP and the I.S. is depicted in panel B. NAP and I.S. are shown to be well recovered and separated. In addition, a small peak at about 4.5 min was detected. This peak could not be identified. As it does not appear either in a sample prepared without hydrolysis (data not shown) nor with hydrolysis of a blank (panel A of Fig. 1), it can be concluded that it originates from hydrolysis in
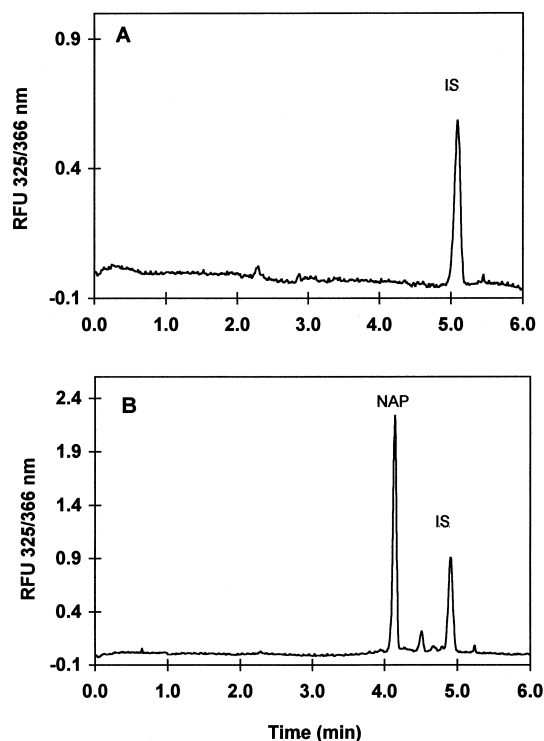

Fig. 1. Electropherograms obtained with extracts of hydrolyzed (A) blank liver homogenate spiked with the I.S. and (B) blank liver homogenate spiked with 2.0 μg/ml NAP (22 μg NAP per gram liver) and I.S. Conditions as described in Section 2.

presence of NAP. However, as is shown below, this peak does not hinder the determination of NAP in tissues of SD rats that were treated with NAP_{23}– HSA or free NAP. It is important to note that LIF solute detection as employed here mainly visualizes the components of interest only while endogenous substances are not detected. This is similar to the conditions employed for MECC of NAP and NAP– protein drug carriers and metabolites in plasma [17] and for MECC based immunochemical drug assays [18].

Typical electropherograms obtained with extracts of hydrolyzed liver homogenates of animals treated with NAP_{23}–HSA and free NAP are depicted in panels A and B of Fig. 2, respectively. In the case of drug targeting with NAP_{23}–HSA (panel A of Fig. 2),

the NAP peak represents total NAP, i.e. NAP originating from the hydrolyzed NAP protein conjugate, the hydrolyzed metabolites and the residual NAP from the drug formulation [17]. Without hydrolysis, the NAP peak would be much smaller (see below). As above, well resolved peaks for both NAP and the I.S. were detected. Furthermore, the electropherogram of Fig. 2A is comparable to that measured with an extract of a hydrolyzed liver homogenate of an animal that was treated with NAP only (panel B of Fig. 2). Similar data were obtained with kidney homogenates (Figs. 3 and 4). Panels A and B of Fig. 3 depict data obtained with extracts of hydrolyzed blank kidney homogenates spiked with I.S. only or with NAP and I.S., respectively, whereas the two panels of Fig. 4 show typical data obtained with kidneys of treated animals. In analogy to the case with liver tissue samples, all electropherograms
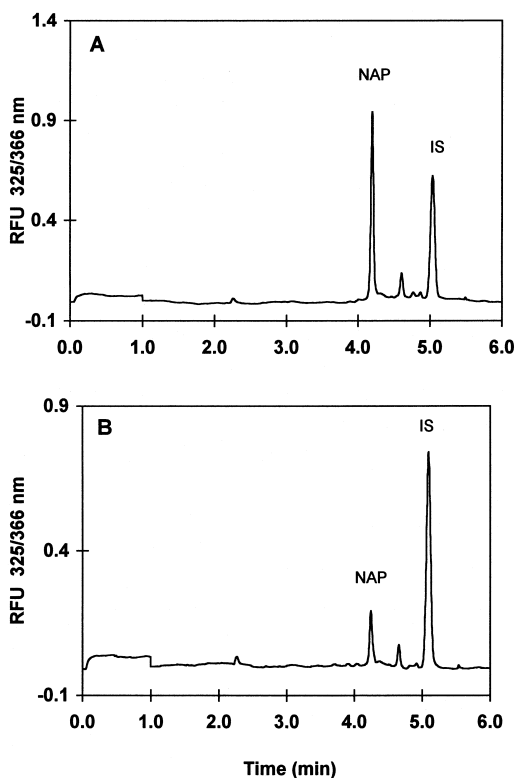

Fig. 2. Electropherograms obtained with extracts of hydrolyzed homogenates of livers that were removed 15 min after administration of (A) 22 mg NAP_{23}–HSA per kg body mass and (B) 1.5 mg NAP per kg body mass. Conditions as described in Section 2. Total NAP drug levels were determined to be (A) 1.51 μg/ml of hepatic tissue homogenate (17.0 μg NAP per g liver) and (B) 0.27 μg/ml of hepatic tissue homogenate (3.0 μg NAP per gram liver).
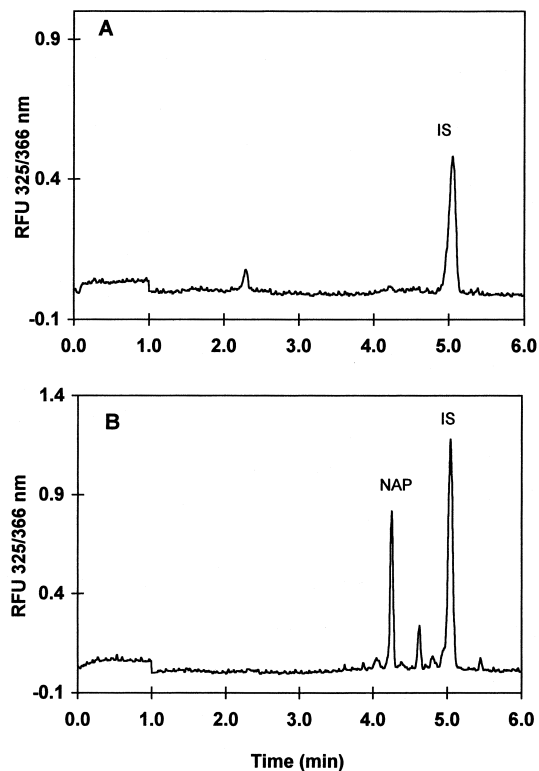

Fig. 3. Electropherograms obtained with extracts of hydrolyzed (A) blank kidney homogenate spiked with the I.S. and (B) blank kidney homogenate spiked with 0.5 μg/ml NAP (3.0 μg NAP per gram kidney) and the I.S. Conditions as described in Section 2.
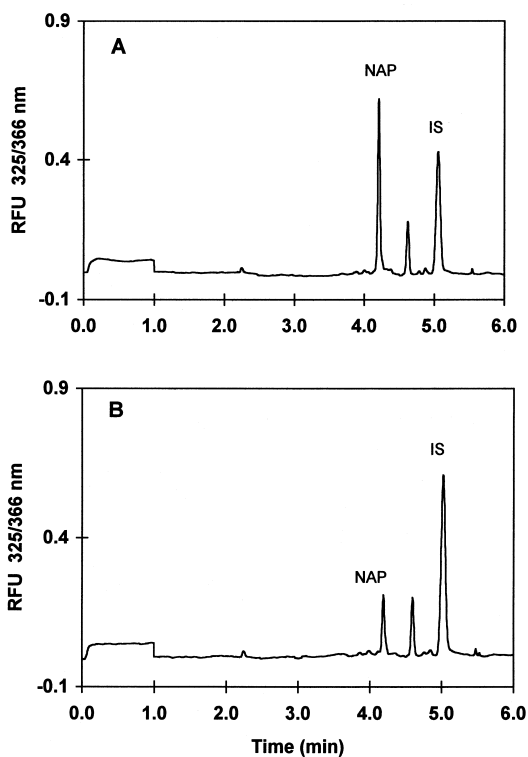
Fig. 4. Electropherograms obtained with extracts of hydrolyzed homogenates of kidneys that were removed 15 min after administration of (A) 22 mg NAP_{23}–HSA per kg body mass and (B) 1.5 mg NAP per kg body mass. Conditions as described in Section 2. Total NAP levels were determined to be (A) 1.41 μg/ml of renal tissue homogenate (8.34 μg NAP per gram kidney) and (B) 0.35 μg/ml of renal tissue homogenate (2.13 μg NAP per gram kidney).

showing a peak for NAP also have the additional, unknown peak between those of NAP and I.S..

3.2. Determination of NAP in extracts of hydrolyzed tissue homogenates

Quantitation of NAP was based upon internal, four- to five-level calibration using the peak height ratio of the compound to the I.S. and having a 0.1–2.0 μg/ml (0.43–8.68 μM) concentration range. During the course of this work, five calibrations with liver homogenate and two calibrations with renal tissue were undertaken. All calibration graphs were determined to be linear with F values>630 ($P<$ 0.0001). The y-intercepts were observed to be significantly smaller than the smallest calibrator values

and were thus negligible. Furthermore, no significant difference between calibrations in hepatic and renal tissue homogenates were noted. Thus, calibration graphs with liver homogenates were typically employed for the assessment of drug levels in both organs. Having a NAP concentration of 0.25 μg/ml, intra-day and inter-day relative standard deviations (R.S.D.) were determined to be 3.73% ($n=9$) and 6.39% ($n=5$), respectively. With the assay conditions described in Section 2, the detection limit (S/N=3) was found to be about 0.07 μg/ml. This sensitivity was found to be sufficient for the determination of the NAP equivalents in liver and kidney tissues harvested 15 min after injection of 22 mg Nap_{23}–HSA per kg body mass or 1.5 mg NAP per kg body mass (Figs. 2 and 4) and for the assessment of the pharmacokinetics of NAP_{23}–HSA and NAP in liver and kidney tissues [19].

In the animal experiments performed, equivalent amounts of NAP were administered via i.v. bolus injection of the two drugs (22 mg Nap_{23}–HSA per kg body mass or 1.5 mg NAP per kg body mass, corresponding to about 6.5 μmol NAP per kg body mass each). Quantitation of the data presented in Figs. 2 and 4 revealed higher total NAP levels in both organs when Nap_{23}–HSA was injected. The NAP content in the liver was found to be 5.7-fold higher compared to that after administration of NAP (Fig. 2, [19]). The drug level increase in the kidneys was found to be lower (Fig. 4, [19]).

3.3. MECC analysis of centrifuged and filtered tissue homogenates

In an attempt to simultaneously monitor free NAP, NAP_{23}–HSA and NAPLYS (metabolite of NAP_{23}–HSA [17]), tissue homogenates containing NAP_{23}–HSA were not hydrolyzed but centrifuged at 16 000 g and filtered with a 0.2-μm syringe filter prior to the addition of the I.S. Analysis of these samples in the same MECC configuration as described above revealed small peaks that could unambiguously be allocated to NAP and NAPLYS. No peak for the protein conjugate was detected. It is known from our previous investigations dealing with direct plasma injection that NAP, NAP_{23}–HSA and NAPLYS separate well under the given MECC conditions [17]. Thus, it appears that NAP_{23}–HSA is attached to the

particulates and is effectively removed by centrifugation and the simplified sample preparation leads to the monitoring of free NAP and NAPLYS only. Furthermore, tissue homogenates prepared with SDS (0.2 g/ml, incubation for 10 min at room temperature while vigorously shaking the sample vial) and ultracentrifugation at 90 000 rpm (222 000 to 350 000 g for 30 min at 4°C) before analysis of the supernatant did not provide any meaningful data. No further work was undertaken to analyze NAP_{23}–HSA in the tissues.

4. Conclusions

MECC with LIF detection is shown to provide a selective, simple, reproducible and attractive approach for the assessment of total NAP in liver and kidney tissues of SD rats that have been treated with NAP and NAP_{23}–HSA, a drug targeting preparation with 23 molecules of NAP covalently bound to HSA. No expensive separation columns have to be employed and sample preparation is simple. Furthermore, the high degree of automation combined with short run times of maximal 6 min with the employed CE instrument make this MECC method highly effective and economic. Compared to the previously applied HPLC methods [11], the MECC assay offers the advantage of having lower running costs and a much smaller consumption of organic solvents. Thus, the described MECC assay has been used instead of an HPLC method to assess pharmacokinetics of NAP in liver and kidney tissues of healthy and cirrhotic animals that have been treated with NAP and NAP_{23}–HSA [19]. This represents the first example for which an electrokinetic capillary technique has been applied to elucidate pharmacological parameters in tissues. Although only data obtained with rat tissue samples have been generated thus far, the assay may also be employed for monitoring NAP in human tissue, e.g. liver biopsies. The drug targeting preparation administered represents an exciting new approach in pharmacotherapy but is not yet employed in human studies. Further work is required for the direct monitoring of the drug targeting compound in tissues by MECC.

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