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Head-column field-amplified sample stacking in binary system capillary electrophoresis Preparation of extracts for determination of opioids in microliter amounts of body fluids

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Abstract

Head-column field-amplified sample stacking (head-column FASS) is an efficient, on-line sample concentration technique that can easily provide a sensitivity enhancement of three orders of magnitude. Application of head-column FASS to the capillary electrophoretic analysis of opioid extracts prepared from 20 to 100 μl of human plasma, serum or urine is reported. In the described approach, efficient concentration of cationic opiates from low conductivity extracts of body fluids is effected across a water plug, with separation taking place in a binary buffer comprising 60% (v/v) ethylene glycol, 75 m*M* Na_2HPO_4 and 25 m*M* NaH_2PO_4 (pH 7.9), and detection is effected at 210 nm. Sample extracts are prepared in 55% (v/v) ethylene glycol containing 100 μ*M* H_3PO_4. Application of mixed-mode polymer solid-phase resins is shown to provide extracts that are either too salty or contain quite a large number of endogenous substances that could interfere with certain opioids. Liquid–liquid extraction with hexane, dichloromethane, ethyl acetate and dichloromethane–isopropanol is shown to provide extracts that are sufficiently clean. At a given pH, however, only closely related opioids can be extracted. Using ethyl acetate at alkaline pH, dihydrocodeine and nordihydrocodeine can reproducibly be recovered from 20–100 μl of plasma, serum and urine. Application of head-column FASS and UV absorption detection thereby leads to the determination of ppb concentrations (≥1 ng/ml) of these compounds, an approach that only requires microliter amounts of sample and organic solvents.

Keywords: Field-amplified sample stacking; Sample stacking; Injection methods; Opioids

1. Introduction

Monitoring solutes on the ppb level by capillary electrophoresis (CE) with UV absorption detection is a challenge. Without any preconcentration of solutes, this technology provides access to ppm or higher concentrations only. Among various approaches based upon electrokinetic sample stacking described in the literature, head-column field-amplified sample stacking (head-column FASS) has been shown to provide the highest sensitivity enhancement [1–4]. Head-column FASS has no limited sample injection volume and sensitivity enhancement of over three orders of magnitude is typically attained. Large amounts of solutes are electrophoretically injected into the capillary without introduction of a significant amount of sample solvent. Head-column FASS is based upon the presence of a short (~mm length)

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zone of low conductivity (mostly, water is used and the zone is referred to as a water plug) at the capillary inlet end, across which an electric field up to several hundred times higher than that employed in normal CE is established and which permits charged analytes to be injected at high velocity. During this electrokinetic injection process, analytes are condensed at the interface between the low-conductivity zone and the running buffer. It has been shown that this injection process can further be optimized by having a short preinjection plug of high ionic strength, high pH and high viscosity [3]. Furthermore, it has been demonstrated that the highest sensitivity enhancement is obtained with a sample solution of low conductivity and of low viscosity containing 50–100 μ*M* H_3PO_4 [1–3] and that the water plug is the key for attaining the highest reproducibility [4].

Determination of opiates and their metabolites in body fluids is important for pharmacokinetic and pharmacogenetic investigations, assessment of analgesic efficacy, determination of a patient's compliance, and for screening and confirmation of drug abuse. For the monitoring of opiates in body fluids, methods based upon gas chromatography (GC) [5–9] and high-performance liquid chromatography (HPLC) [10–15] are widely used. These methods typically require large amounts of biological samples (1–5 ml). CE has recently been shown to represent a simple and low-cost approach for monitoring urinary opiates [16–22]. Using large volumes of urine and off-line preconcentration via extraction, the lowest detectable concentration (LDC) has been determined to be between 0.01 and 1 μg/ml. This sensitivity is not sufficient for the measurement of opiates in small amounts of blood, as the drug levels in serum or plasma are typically <0.2 μg/ml and assays based upon the use of several ml of blood are unattractive and inconvenient. For investigations requiring multiple blood samples to be taken from an individual, drawing of several ml at each time point can often not be justified or might even be impossible, as in the case of assessing drug levels in samples from infants and children.

Model mixtures of opiates have been shown to separate nicely by CE in micellar and binary media [23]. Furthermore, using head-column FASS and binary CE with ethylene glycol, these opiates can be analyzed reproducibly on the ppb level using internal calibration. Intra- and inter-day RSD values for the 3 to 10 ng/ml concentration levels are typically <5% and the LDC for dihydrocodeine (DHC) and its metabolites is 0.1 ng/ml (~0.3 n*M*) [3]. The most important prerequisite for the achievement of the high sensitivity associated with head-column FASS is that samples have to be free of electrolytes. Thus, biological samples have to be pretreated. Liquid–liquid extraction of very hydrophobic amiodarone-like compounds from human serum using hexane has been shown to provide extracts that are clean enough for implementation of head-column FASS [2]. Opiates are slightly hydrophobic compounds and their extraction behavior is therefore significantly different to that of compounds that are extractable with hexane or another non-polar solvents.

The work described here is a continuation of our previous efforts in developing robust approaches to head-column FASS [1–4]. The goals comprised investigation of the conditions for extraction of moderately hydrophobic opioids, including DHC and its major metabolites, from μl amounts of human plasma, serum and urine, followed by analysis of the extracts by head-column FASS and binary CE with ethylene glycol. The application of various solid-phase and liquid–liquid extraction schemes is discussed.

2. Experimental

2.1. Drugs and chemicals

DHC and its metabolites nordihydrocodeine (NDHC), dihydromorphine (DHM) and nordihydromorphine (NDHM) were received from Mundipharma (Basel, Switzerland). Norcodeine (NCOD) and normorphine (NMOR) were purchased as methanolic solutions (1.0 mg/ml base) from Alltech (State College, PA, USA). Methadone (MET) hydrochloride was of European Pharmacopoeia quality and supplied by the university hospital pharmacy (Berne, Switzerland). Dextromethorphan hydrobromide was a kind gift from F. Hoffmann-La Roche (Basel, Switzerland). Ethylene glycol was purchased from Merck (Darmstadt, Germany). β-Glucuronidase (G7646; from *Escherichia*

coli) was from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Subjects, plasma and urine samples, blank matrices and standard solutions

Plasma samples originated from healthy volunteers who gave their consent, ingested 60 mg of DHC in the form of a slow release tablet (DHC Continus tablet containing 90 mg of DHC hydrogentartrate; Napp Labs., Cambridge, UK) and participated in a controlled study [22]. On a separate day, the same subjects ingested 50 mg of quinidine sulfate (capsules manufactured by the university hospital pharmacy, Berne, Switzerland) 2 h prior to swallowing the same amount of DHC. Sera from patients under theophylline pharmacotherapy that were received for therapeutic drug monitoring, plasma from the volunteers taken before administration of DHC or our own plasma were used as blank matrices. Urine samples were those collected during the 0–8 h time interval after administration of 25 mg of DHC (75 drops of Paracodin, Knoll, Liestal, Switzerland). Our own urine was employed as a blank. All samples were stored at −20°C. Aqueous stock solutions of drugs (1 mg/ml) were prepared with 2 m*M* H_3PO_4. Standard solutions (50–5000 ng/ml) were prepared by diluting appropriate aliquots of the stock solutions with water. All solutions were stored at 4°C in plastic vials.

2.3. Sample preparation

For enzymatic hydrolysis, 100 μl of plasma/urine, 10 μl of internal standard solution, 20 μl of β-glucuronidase solution (100 U) and 80 μl of 0.1 *M* phosphate buffer (pH 6.8) were vortex-mixed and incubated at 37°C for 17 h. Solid-phase extraction was effected using disposable, mixed-mode polymer cartridges (Bond Elut Certify, No. 1211-3050) and the Vac-Elut set-up (both from Analytichem International, Harbor City, CA, USA). The cartridges were conditioned as summarized in Table 1 using vacuum aspiration without drying the sorbent bed. Aliquots of plasma/urine or hydrolysate, standard solutions and carbonate buffer, pH 9 (0.3 *M* $NaHCO_3$–0.04 *M* NaOH) were mixed (see Table 1), loaded onto and slowly drawn through the cartridges. Prior to elution of the adsorbed opioids with 1.5 to 2 ml of solvent, the cartridges were sequentially washed with various solutions while applying vacuum aspiration (no drying; Table 1). The first five drops of the eluates were discarded and the rest of the eluates were collected and evaporated to dryness in open glass tubes (at 37°C for 20 min under a gentle stream of nitrogen or for four days without a stream of nitrogen). Prior to analysis, the residues were redissolved in 100 μl of sample solvent. For liquid–liquid extraction, aliquots of plasma/urine or hydrolysed plasma, Na_2CO_3 buffer and one or several organic solvent(s) (Table 2) were vortex-mixed for 1 min in a plastic vial and centrifuged at 9000 *g* for 20–30 s prior to transferring part of the organic phase into a 0.35 or 1.0 ml glass tube and evaporated to dryness. The residue was redissolved in 100 μl of sample solvent (55%, v/v, ethylene glycol containing 100 μ*M* H_3PO_4) and then transferred into a 0.5-ml plastic sample vial that could be placed into the autosampler of the CE apparatus.

2.4. CE analysis

CE was performed on an ABI 270A-HT capillary electrophoresis system (Applied Biosystems, Foster City, CA, USA) equipped with a 41-cm (22 cm effective length)×50 μm I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA). A new capillary was first flushed with 1 *M* NaOH for about 30 min and capillary conditioning between runs was effected by flushing with running buffer for 5 min (application of a vacuum of 20 in.Hg at outlet end of the capillary) or sequentially with 0.1 *M* NaOH (3 min), water (2 min) and running buffer (2 min) (1 in.Hg=338.638 Pa). The running buffer was composed of 60% (v/v) ethylene glycol, 75 m*M* Na_2HPO_4 and 25 m*M* NaH_2PO_4 (pH 7.9). The inlet buffer vial was filled with the running buffer whereas the outlet vial contained an aqueous buffer comprising 30 m*M* Na_2HPO_4 and 10 m*M* NaH_2PO_4. If not stated otherwise, a preinjection plug of 3.1 mm length (application of 5 in.Hg vacuum for 4.0 s) composed of 90% (v/v) ethylene glycol and 0.2 *M* Na_3PO_4 was applied at the inlet side [3]. The capillary tip was dipped for 6 s into a vial containing water. This has two effects. It cleans the capillary tip and a short water plug is inserted into the capillary

Table 1
Solid-phase extraction procedures with Bond Elut Certify for DHC and NDHC in body fluids[a]

		Extraction procedure					
		I	II	III	IV	V	VI
		Elution solvent					
		Methanol 30% NH_3	Methanol 30% NH_3	Dichloromethane–isopropanol (8:2), 2% NH_3	Dichloromethane–isopropanol (8:2. v/v), 2% NH_3	Dichloromethane–isopropanol (8:2, v/v), 2% NH_3	Dichloromethane–isopropanol (8:2, v/v), 2% NH_3
Conditioning steps:	(1) Methanol (ml)	5	2	5	5	5	2
	(2) Water (ml)	5	2	5	5	5	2
	(3) Carbonate buffer[b], pH 9 (ml)	1	–	1	–	–	–
Washing steps:	(1) Carbonate buffer[b], pH 9 (ml)	1	–	1	1	–	–
	(2) Water (ml)	2	2	2	2	–	2
	(3) 0.1 *M* acetate buffer (pH 4) (ml)	1	1	1	1	–	1
	(4) Water (ml)	5 (4 times)	–	5 (4 times)	5 (4 times)	5 (4 times)	–
	(5) Methanol (ml)	2	2	2	2	2	2
Conductivity of diluted sample[c] (Ω^{-1} m^{-1})		0.0017	ND	0.0003–0.0004	0.0004	0.0003	ND

[a] Without hydrolysis, the sample applied comprised 450 μl of a mixture composed of 100 μl of plasma/urine, 10 μl of standard solution, 400 μl of 0.3 *M* $NaHCO_3$–0.04 *M* NaOH (pH 9). With hydrolysis, 900 μl of a mixture composed of 210 μl of plasma/urine hydrolysate (cf. Experimental) and 800 μl of 0.3 *M* $NaHCO_3$–0.04 *M* NaOH (pH 9) was applied. For system I, the eluate was dried at 37°C (for four days) and reconstituted in 100 μl of sample solvent. For systems II to VI, eluates were dried within 20 min at 37°C under a gentle stream of nitrogen and reconstituted in 100 μl of sample solvent.

[b] Buffer was composed of 0.3 *M* $NaHCO_3$ and 0.04 *M* NaOH.

[c] Conductivity was determined by diluting 60 μl of sample (plasma extract prepared in sample solvent) with 4940 μl of water.

Table 2
Liquid–liquid extraction procedures for DHC and NDHC in body fluids

	Extraction procedure						
	I	II	III	IV	V	VI[d]	VII
	Extraction solvent(s)						
	Hexane	Dichloro-methane	Dichloro-methane	Dichloro-methane–isopropanol (9:1, v/v)	Ethyl acetate	Ethyl acetate	Ethyl acetate
	Extraction pH[c]						
	ND	ND	10.3	10.3	10.6	10.3	10.3
Plasma, serum or urine (μl)	100	20	100	100	20	100	100
I.S. concentration (ng/ml)	–	500	150	150	500	150	30
Volume of I.S. solution (μl)	–	10	10	10	10	10	10
Volume (conc.) of carbonate solution (μl)	20 (1.2 *M*)	10 (0.2 *M*)	10 (2 *M*)	10 (2 *M*)	10 (0.5 *M*)	10 (2 *M*)	10 (2 *M*)
pH of Na_2CO_3 solution	10.4	11.8	11.4	11.4	11.8	11.4	11.4
Volume of organic solvent (μl)	200	50	150	150	50	150	150
Volume of plastic vial used for extraction (ml)	0.5	0.2	0.4	0.4	0.2	0.4	0.4
Volume of transferred organic phase (μl)	100	20	120	120	20	120	120
Volume of glass vial used for evaporation (ml)	0.35	0.35	1	1	0.35	1	1
Evaporation temperature (°C)/gas flow	RT[b]/–	RT[b]/–	37/air	37/air	RT[b]/–	37/N_2	37/N_2
Evaporation time (min)	≈60	≈20	20	20	120	5	5
Sample solvent[a] volume for reconstitution (μl)	100	100	100	100	100	100	100

[a] Sample solvent: 55% (v/v) ethylene glycol containing 100 μ*M* H_3PO_4.
[b] RT=room temperature.
[c] pH of plasma and serum after addition of I.S. and carbonate solution.
[d] With hydrolysis, 210 μl of plasma hydrolysate (cf. Experimental), 20μl of 2 *M* Na_2CO_3 and 290 μl of ethyl acetate were mixed and centrifuged. A 250-μl volume of the organic phase was transferred to a 1-ml glass vial, evaporated to dryness and reconstituted in 100 μl of sample solvent.

[4]. Additionally, a water plug of 0.6–0.8 mm length (application of 5-inch Hg for 1.0 s) was introduced into the capillary. Injection of sample occurred using a voltage of 10 kV (anode on the injection end) for 99.9 s (current, 13–7 μA). The run voltage was 23 kV (anode on the injection end). Due to the low conductivity of the sample zone, the current gradually increased from about 22 to 41 μA within the first 30 s of power application and then slowly reached a level of about 48 μA. Solute detection was effected by UV absorbance at 210 nm. A PC integration pack (version 3.0, Kontron Instruments, Zurich, Switzerland) together with a 486 computer system was used for data collection, handling and storage. Quantification was based on five-level internal calibration employing peak heights.

2.5. Conductivity and pH determinations

The conductivity was measured with a conductivity meter, model 101 (Orion Research, Cambridge, MA, USA), equipped with a model PW 9510/65 cell (Philips, Eindhoven, Netherlands). For pH measurement, a pH meter, model 720, and a ROSS pH electrode, model 8103 (both from Orion Research) were used.

3. Results and discussion

3.1. CE of opioids using head-column FASS

As discussed elsewhere in detail [3], the application of head-column FASS to the analysis of opioids is straightforward and leads to the analysis of these compounds at the n*M* or ppb concentration level. This approach can be reliably operated with a water plug only [3,4] or with a preinjection ion trap (preinjection plug) in front of the water plug, which was found to provide about a two-fold increase in

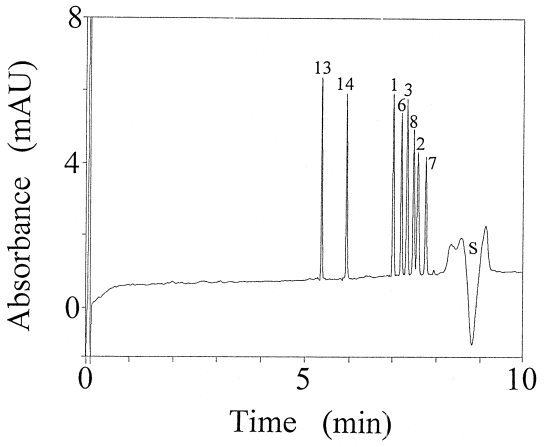

Fig. 1. Separation of opioids in standard sample (55% ethylene glycol containing 100 μ*M* phosphoric acid) by CE with head-column FASS. The solute concentrations were 20 ng/ml for methadone (13), 40 ng/ml for dextromethorphan (14) and 10 ng/ml for NDHC (1), NCOD (2), DHC (3), NDHM (6), NMOR (7) and DHM (8). S marks the sample solvent peak. Other conditions are given in Section 2.4.

sensitivity [3]. The data presented in Fig. 1 show that DHC and its metabolites NDHC, NDHM and DHM separate well under the conditions described. Furthermore, NCOD and NMOR were found not to interfere. The same was true for MOR and COD, compounds that elute just ahead of the sample solvent [3]. Furthermore, methadone and dextromethorphan (potential candidates for use as internal standards) were determined to migrate in front of all of the opioids. A precondition for implementation of the electrokinetic stacking technique with high sensitivity is that the sample has to be free of electrolytes. Thus, the salts in biological samples have to be removed, e.g., via selective extraction of the compounds of interest. Preparation of suitable extracts by solid-phase and liquid–liquid extraction procedures are discussed in turn.

3.2. Preparation of extracts by solid-phase extraction

Solid-phase extraction of opioids in body fluids has become popular in recent years [11–22]. A mixed-mode polymer phase was successfully employed in our laboratory for extraction of a wide range of urinary opioids prior to their analysis by micellar electrokinetic capillary chromatography (MECC) [16–19,21,22,24]. Starting with 5 ml of urine, this approach allowed the simultaneous determination of morphinoids and codeinoids with a detection limit of about 50 ng/ml [21]. Thus, plasma and urine extracts prepared with the Bond Elut Certify cartridges (Table 1) were applied to head-column FASS for analysis of ppb drug concentrations in 100 μl of body fluids. Typical electropherograms are presented in Figs. 2 and 3.

Using an elution with methanol that contains 30% (v/v) concentrated ammonia (system I of Table 1) provided an opioid-containing extract that could not be analyzed by CE with head-column FASS. The same was found to be true after extract preparation with less conditioning and washing steps (system II of Table 1; for a typical electropherogram, see insert of Fig. 3B) or with cartridges containing another mixed-mode polymer phase (Bond Elut Certify II, Analytichem International; data not shown). Furthermore, when the residue after solid-phase extraction was redissolved in the standard sample (Fig. 1), no peaks could be monitored. This confirmed that residual amounts of electrolytes originating from elution completely destroyed the stacking efficiency. Not surprisingly, the conductivity of the reconstituted sample was determined to be much higher than the conductivity of the standard sample of Fig. 1 (0.0017 vs. 0.0003 Ω^{-1} m^{-1}, respectively). For measuring conductivities, 60 μl of sample were diluted to 5 ml with bidistilled water. In that way, bidistilled water and running buffer were determined to have conductivities of 0.0002 and 0.0251 Ω^{-1} m^{-1}, respectively. In an approach to assess the amount of ions required to make head-column FASS unfunctional, carbonate buffer was added to the standard sample (Fig. 1) prior to reanalysis of the modified sample. Having 10 or 20 μ*M* Na_2CO_3 in the sample, head-column FASS was possible. However, with≥50 μ*M* Na_2CO_3, no electrophoretic peaks were observed. It is important to note that one paper reports the determination of drugs in urine after solid-phase extraction with C_{18} cartridges and subsequent analysis of the extract by CE using head-column FASS. In this approach, a loss of sensitivity is actually obtained. The sensitivity was determined to be 20-fold lower compared to that observed with the standard sample [25,26]. This difference can

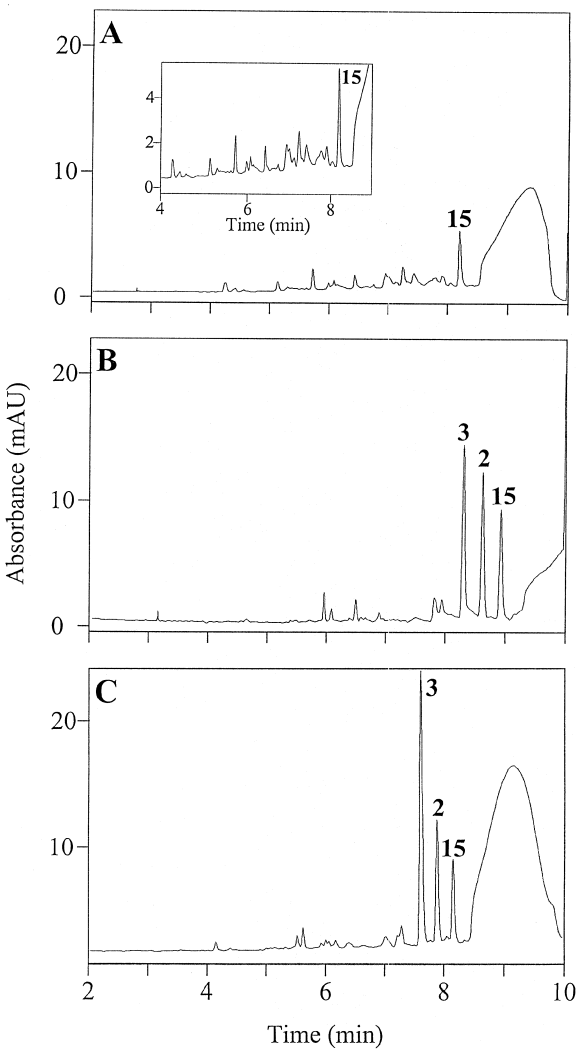

Fig. 2. Electropherograms obtained after solid-phase extraction (system IV of Table 1) of (A) plasma from a subject, which was drawn 2 h after ingestion of 50 mg of quinidine sulfate and just before administration of DHC, (B) plasma (from the same subject) taken 3.25 h after administration of 60 mg of DHC and (C) hydrolysed plasma (same sample as in panel B). For the data of panels B and C, 150 ng/ml NCOD were added to the plasma. The inset in panel A depicts a segment of the blank's baseline on an elongated *y*-axis scale. All samples were reconstituted in 100 μl of sample solvent and injected with head-column FASS. No preinjection plug was used. DHC concentrations in B and C were determined to be 170 and 360 ng/ml, respectively. Key: 2, NCOD; 3, DHC; 15, quinidine.

certainly be ascribed to the presence of electrolytes originating from the sample clean-up process.

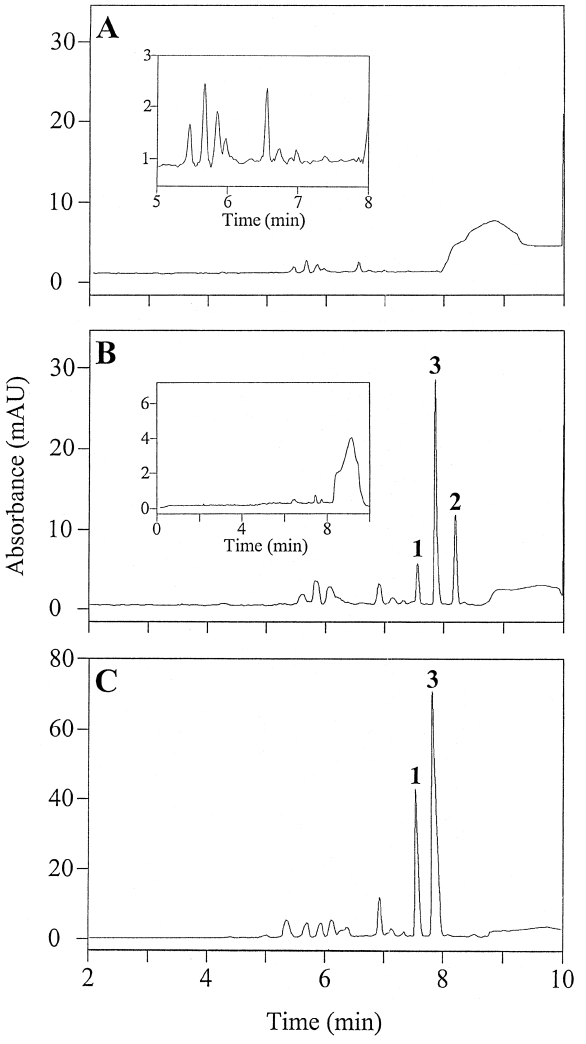

Fig. 3. Analysis of urinary opioids by CE with head-column FASS after solid-phase extraction (system VI of Table 1) of (A) blank urine, (B) blank urine fortified with 80 ng/ml NDHC, 500 ng/ml DHC and 150 ng/ml NCOD and (C) 0–8 h urine of a subject who ingested 25 mg of DHC. The inset in panel A depicts a segment of the blank's baseline on an elongated *y*-axis scale. The inset in panel B depicts data obtained with extraction scheme II of Table 1. Other conditions were the same as for Fig. 2. Key: 1, NDHC; 2, NCOD; 3, DHC.

For the performance of head-column FASS without loss of stacking efficiency, alternate elution procedures were evaluated. Using dichloromethane–isopropanol (8:2, v/v) containing 2% concentrated ammonia solution instead of alkalinized methanol as

eluent provided extracts that had lower conductivities (systems III–VI of Table 1) and could be analyzed by head-column FASS. The various systems investigated differ mainly in terms of cartridge conditioning and the wash steps prior to opioid elution. Using DHC, NDHC and NCOD as test compounds, system V provided insufficient opioid recovery. All other configurations resulted in acceptable data. Thus, conditioning with carbonate buffer, pH 9 (system III) and the washing steps with water and carbonate buffer (but not the wash with the acetate buffer that was abolished in system V) were found to be unnecessary. Typical data obtained with systems IV and VI are presented in Figs. 2 and 3, respectively.

For extraction of blank plasma, blank plasma spiked with the opioids and a volunteer's plasma drawn after administration of DHC using systems III to V, the conductivities of the reconstituted and diluted samples were determined to be around 0.0004 Ω^{-1} m^{-1}. This value is somewhat higher than that obtained after liquid–liquid extraction with ethyl acetate (0.0003 Ω^{-1} m^{-1}, conditions of procedure V of Table 2, see below) but were much lower than the 0.0017 Ω^{-1} m^{-1} determined for the extract after elution with alkalinized methanol. Electropherograms revealed a number of endogenous peaks that migrated ahead of and around the compounds of interest (Fig. 2A). Analysis of plasma that was spiked with 11 opioids at about 100 ng/ml each provided electropherograms that were significantly more complex compared to those shown in Fig. 2 (data not shown). Thus, it was difficult to allocate the peaks to the various opioids. Having samples from patients containing DHC, NDHC and NCOD, however, peak assignment of compounds whose concentrations were >40 ng/ml could be executed unambiguously (Fig. 2B). The same was found to be true after plasma hydrolysis (Fig. 2C). It is known that the analyzed samples also contained about 10 to 20 ng/ml NDHC, as well as small amounts of DHM and NDHM [27]. However, these compounds could not be determined in these plasma extracts. Similar observations were made for analysis of urinary extracts by CE with head-column FASS (Fig. 3). For plasma DHC, internal calibration in the 20–500 ng/ml range (150 ng/ml NCOD as internal standard) was found to be linear ($r=0.989$). The y-intercept was 34 ng/ml, indicating that the determination of DHC concentrations below 40 ng/ml cannot be performed accurately. Identification limits for analysis of opioids in solid-phase extracts of 100 μl of body fluid as described here were found to be ≥40 ng/ml and intra-day reproducibility was found to be unsatisfactory. At 100 ng/ml NDHC and DHC drug concentrations, RSD values up to 32% were observed ($n=10$).

3.3. Preparation of extracts by liquid–liquid extraction

The various extraction procedures investigated are summarized in Table 2. In previous work, liquid–liquid extracts of amiodarone from serum using hexane were shown to be clean enough for application to head-column FASS [2]. With this solvent at alkaline pH, the somewhat hydrophilic opioids could not, whereas methadone and dextromethorphan could, be extracted (Fig. 4A, column I in Table 2). With cylcohexane, a similar extraction behavior was observed (data not shown). Polar solvents, such as halogenated hydrocarbons mixed with alcohols, have been widely used for liquid–liquid extraction of opioids from biological samples [5–10]. Using 50 μl of dichloromethane (or chloroform) and 20 μl of body fluid (column II of Table 2) did not provide any meaningful head-column FASS CE data. Electrolytes (>50 μ*M*, see above) in the sample presumably prevented proper stacking of the solutes of interest. Difficulties in transferring the small amount of the organic (lower) phase without carry-over of electrolytes from the aqueous (upper) phase were encountered. Starting with 100 μl of plasma and 150 μl of organic solvent (columns III and IV of Table 2), however, nice data were obtained (Fig. 5A and B). The same was found to be true using ethyl acetate (Fig. 5C), a solvent that is lighter than water and can thus more easily be transferred without contaminating the extract. Some of the opioids, but not all, were found to extract well with ethyl acetate and the extracts were determined to be applicable to head-column FASS (Fig. 4B–D). Not surprisingly, the recoveries of NDHC, NCOD and DHM were found to be strongly dependent on the extraction pH (Fig. 4B–D), whereas the recovery of DHC changed very little over the pH range investigated. NDHC and NCOD were determined to extract better at

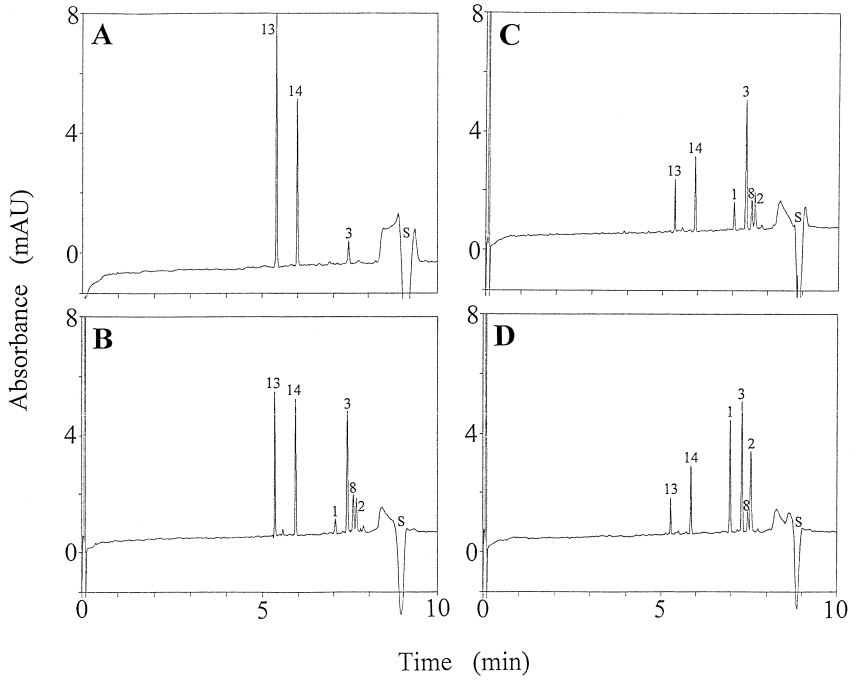

Fig. 4. Data obtained with different liquid–liquid extraction procedures using (A) hexane (system I of Table 2) and (B,C,D) ethyl acetate [similar to system V of Table 2; the extraction pH values being 7.4 (no buffer added), about 8.9 (addition of carbonate buffer, pH 8.9) and about 11 (addition of carbonate buffer, pH 11.8), respectively]. The solute concentrations in the spiked sera were (A) 100 ng/ml methadone, 200 ng/ml dextromethorphan and 50 ng/ml each of NDHC, NDHM, DHC and DHM, and (B,C,D) 500 ng/ml methadone, 1000 ng/ml dextromethorphan and 250 ng/ml of each opioid. Peak numbers are the same as in Fig. 1. Other conditions as for Fig. 1.

elevated pH, at which they are becoming neutral molecules. These findings are similar to those found in the literature [7] in which COD is reported to extract well in the pH range 8–11, whereas the recovery of NCOD is shown to improve as the pH is increased. Unfortunately, the extraction recovery of DHM decreases as the pH is increased. The morphinoids become negatively charged at pH>10 [23] and, thus, their solubilities in the aqueous phase increase. The optimal pH for extraction of MOR and NMOR have been determined to be 8.96 [9] and about 10 [7], respectively, values that are close to their isoelectric points (MOR, 9.1 and NMOR, 9.3) [23]. NDHM and NMOR could not be extracted with ethyl acetate. Compared to the other opioids, lower extraction recoveries have also been reported for these *N*-demethylated morphinoids using procedures based upon liquid–liquid [7,9] and solid-phase extraction [11,21]. It can be concluded that there is no universal recipe for liquid–liquid extraction that produces suitable low conductivity extracts for head-column FASS of DHC and all of its major metabolites.

Extraction procedures VI and VII were used for analysis of DHC, NDHC and NCOD in plasma (Fig. 6) and urine (Fig. 7). Compared to solid-phase extraction (Figs. 2 and 3), much cleaner electropherograms were obtained. Consequently, identification limits of about 5 ng/ml can be reached. Using this approach, NDHC concentrations of 10 to 20 ng/ml in plasma and hydrolysed plasma can be analyzed (Fig. 6B and C, respectively). The use of a high pH for extraction favored the recovery of NDHC. At that pH, NCOD was found to extract better than methadone and dextromethorphan and was therefore used as the internal standard for the determination of DHC and NDHC. Using procedure V (Table 2), the recoveries for NDHC and DHC

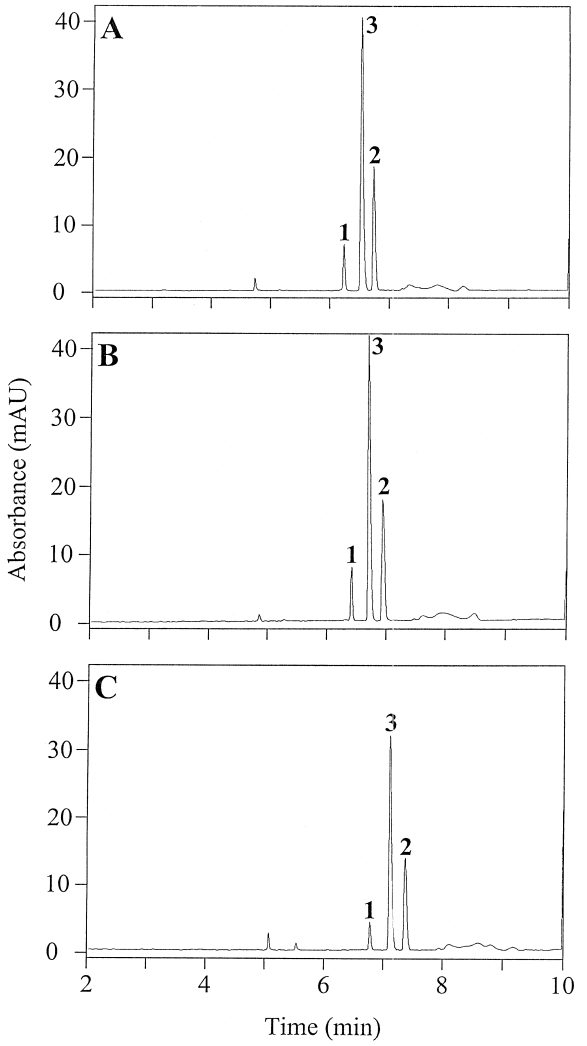

Fig. 5. Data obtained after liquid–liquid extraction of plasma fortified with DHC (400 ng/ml), NDHC (60 ng/ml), NCOD (150 ng/ml) and MET (60 ng/ml) using extraction system (A) III, (B) IV and (C) VI of Table 2. Other conditions as for Fig. 2. Key: 1, NDHC; 2, NCOD; 3, DHC. MET could not be assigned.

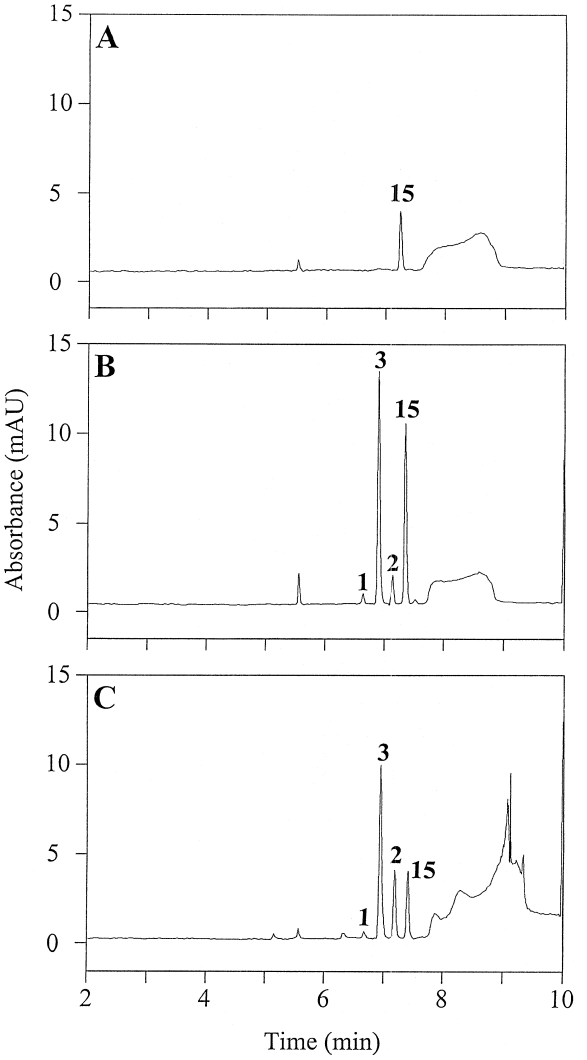

Fig. 6. Data obtained with the plasma samples as for Fig. 2 but using liquid–liquid extraction with ethyl acetate. Electropherograms of (A) blank plasma, (B) unhydrolysed plasma and (C) hydrolysed plasma are shown. For the data presented in panels B and C, 30 and 150 ng/ml of NCOD were added, respectively. Extraction was according to systems VI, VII and VI of Table 2, respectively. All other conditions were the same as for Fig. 2. Key: 1, NDHC; 2, NCOD; 3, DHC; 15, quinidine.

(plasma levels of 25–500 ng/ml) were determined to be 43 and 65%, respectively. The recoveries of NCOD and DHM were about 50 and 8%, respectively, and those for NDHM and NMOR were <2%. After extraction according to procedure VI (Table 2), recoveries for NDHC and DHC were found to be 47 and 70%, respectively. All recovery data were evaluated based on peak heights.

3.4. Quantification of DHC and NDHC in human plasma

Plasma samples drawn up to 13.5 h after DHC ingestion were analyzed using extraction procedure VII (Table 2). In all samples, DHC and its metabo-

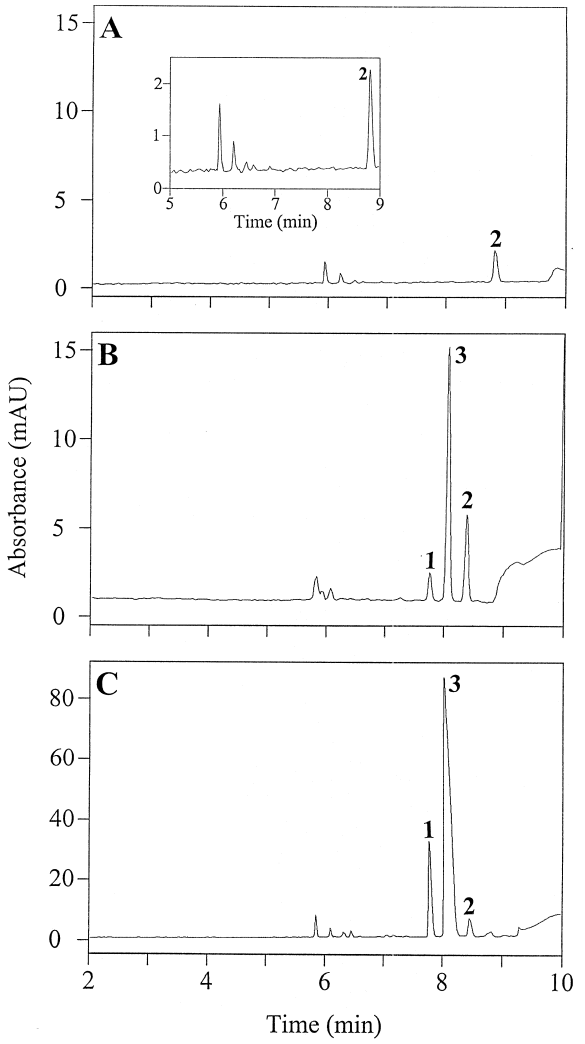

Fig. 7. Electropherograms of the urine samples of Fig. 3 obtained after liquid–liquid extraction with ethyl acetate (system VI of Table 2). The inset in panel A depicts part of the data with an elongated *y*-axis scale. NCOD (150 ng/ml) was used as an internal standard. Other conditions were the same as for Fig. 2. Key: 1, NDHC; 2, NCOD; 3, DHC.

lite NDHC could be monitored using head-column FASS with a 6-s water dip and introduction of a 0.6-mm water plug (1 s) (see Section 2.4). Quantification was based on internal calibration using peak heights. Five calibrators prepared from spiked plasma covering a concentration range of 10–300 and 5–40 ng/ml for DHC and NDHC, respectively, were employed together with NCOD (30 ng/ml) as internal standard. Intercepts and correlation coefficients were 1.12 ng/ml and 0.991, and 0.10 ng/ml and 0.997, respectively, and the limits of identification were 4 and 5 ng/ml, respectively. Reproducibility was assessed by analysis of spiked plasma samples at a level of 100 ng/ml DHC and 20 ng/ml NDHC. Typical intra- and inter-day imprecisions ($n=5$) were found to be <15%. DHC and NDHC concentrations were determined to be between 25 and 220 ng/ml (mean, 93.2 ng/ml; *n*, 55) and 4.4–24 ng/ml (mean, 11.0 ng/ml; *n*, 55), respectively. The same set of samples was also analyzed after enzymatic hydrolysis (cf. Section 2.3) employing extraction procedure VI modified to handle plasma hydrolysates (Table 2). Calibration was carried out using five calibrators in the ranges of 20–500 ng/ml and 10–80 ng/ml for DHC and NDHC, respectively. Data evaluation based on peak heights revealed intercepts and correlation coefficients of 9.4 ng/ml and 0.995 (DHC), and 3.8 ng/ml and 0.999 (NDHC), respectively. Reproducibility was assessed by analysis of spiked plasma samples at a level of 150 ng/ml DHC and 20 ng/ml NDHC. Typical intra- and inter-day RSD values ($n=5$) were found to be <15%. The calculated values for DHC and NDHC were between 100 and 487 ng/ml (mean, 281 ng/ml; *n*, 55) and 12–147 ng/ml (mean, 37.1 ng/ml; *n*, 55), respectively. The mean plasma values for DHC and NDHC are approximately three times as high as the values found for the free substances in unhydrolysed plasma, indicating that DHC and NDHC are conjugated to about the same extent. This is in contrast to the findings in urine in which a higher percentage of DHC compared to NDHC was found to be conjugated [21]. As no glucuronide standards were available, the efficiency of the hydrolysis could not be tested. The procedure employed is similar to that used elsewhere for which the hydrolysis efficacy of DHC-6-glucuronide was reported to be at the 60% level [27].

4. Conclusions

The data presented illustrate that solid-phase extraction schemes employed previously for the simultaneous extraction of a broad variety of opiates cannot simply be applied to CE with head-column

FASS. Extracts of DHC from human plasma obtained with cartridges containing mixed polymer solid phases and final elution with methanol containing 30% concentrated ammonia contain too much salt. Using methylene chloride–isopropanol (8:2, v/v) with 2% concentrated ammonia for elution, extracts can be applied to head-column FASS but do provide electropherograms that might be difficult to evaluate. Furthermore, DHC and NDHC concentrations <40 ng/ml cannot be assessed using this approach with microliter amounts of plasma and urine. Salts introduced during liquid–liquid extraction with various organic solvents were found not to affect the stacking efficiency. Drug levels of opiates as small as a few ng/ml can be monitored by CE with head-column FASS and UV-absorption detection. Best results were obtained using ethyl acetate. However, as the extraction efficiency is optimized by adjusting the pH, only closely related opiates can be recovered.

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