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Capillary electrophoretic separation, immunochemical recognition and analysis of the diastereomers quinine and quinidine and two quinidine metabolites in body fluids

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Abstract

The capillary electrophoretic separation and immunochemical recognition of the two naturally fluorescing, cationic diastereomers quinine (QN) and quinidine (QD), their hydroderivatives and two major QD metabolites (3-hydroxyquinidine and quinidine-*N*-oxide) was investigated. Plain aqueous phosphate buffers and an alkaline buffer containing dodecyl sulfate micelles are shown to be incapable of resolving the two diastereomers. However, incorporation of an additional chemical equilibrium (with β-cyclodextrin) in the case of capillary zone electrophoresis (CZE) and the presence of a small amount of an organic solvent as buffer modifier (2-propanol) in dodecyl sulfate based micellar electrokinetic capillary chromatography (MECC), were found to provide separation media which lead to complete resolution of QN, QD and the other compounds of interest. Furthermore, for MECC- and CZE-based immunoassay formats, a commercially available antibody against QD was found to be a perfect discriminator between QD and QN. It was determined to recognize QD and the two QD metabolites (cross reactivity of 20–30%) but not QN. MECC and CZE with laser induced fluorescence (LIF) detection are shown to be suitable to determine QD and metabolites in urine and plasma (quinidine-*N*-oxide only) collected after single dose intake of 50 mg QD sulfate and of QN in urine, saliva and serum samples that were collected after self-administration of 0.5 l of quinine water (25 mg of QN). With direct injection of a body fluid, MECC with LIF was found to provide 10 ng/ml detection limits for QD and QN. This ppb sensitivity is comparable to that obtained in HPLC assays that are based upon drug extraction. Furthermore, MECC and CZE assays with UV detection are shown to provide the ppm sensitivity required for therapeutic drug monitoring and clinical toxicology of QD and QN.

Keywords: Capillary electrophoresis; Fluorescence; Immunoassay; Stereoisomers; Quinine; Quinidine; Hydroquinine; Hydroquinidine; Quinidine-*N*-oxide; 3-Hydroxyquinidine; Drugs; Body fluids

1. Introduction

Quinine (QN) and quinidine (QD) are two interesting, naturally fluorescing diastereomers (Fig.

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1) with similar pK_a values (4.1 and 8.5, 4.2 and 8.8, respectively [1]) and UV absorbance and fluorescence properties. QD and QN are the 8R, 9S and 8S, 9R, respectively, stereoisomers of 6′-methoxycinchonan-9-ol. QN is the major alkaloid in cinchona bark. It is used: (i) as antimalaric drug (daily doses of 600–2000 mg, typically as sulfate) in the treatment of chloroquine resistant strains of *Plasmodium falciparum* and in patients with severe and complicated malaria; (ii) for relief of skeletal muscle cramps; and (iii) to provide the bitter taste in soft drinks. In humans, QN is oxidized to several more polar hydroxy metabolites, e.g. 3-hydroxy-quinine and 2′-hydroxy-quinine. The therapeutic plasma concentration range is 3–7 μg/ml and blood concentrations $\geq$10 μg/ml can cause toxic effects and might be lethal. The plasma half life is reported to be 4–15 h (mean: 9 h) and may increase during malarial infection. Plasma protein binding is 70–90%. Furthermore, commercial preparations of QN contain up to 10% of the hydroform of QN (hydroquinine (H-QN), Fig. 1) [1,2]. QD is also present in the bark of various species of Cinchona, but in a lower concentration. It is commonly prepared by isomerization of QN from which it is a dextrorotatory stereoisomer. QD is used as antiarrhythmic drug, which is administrated intravenously or intramuscular (200–750 mg as gluconate) in acute therapy or orally (600–4000 mg daily, usually as sulfate) in maintenance therapy. QD is oxidized in the body to 2′-quinidinone, 3-hydroxyquinidine (3-OH-QD), quinidine-*N*-oxide (QD-*N*-OX) and quinidine-10,11-dihydrodiol. The concentration of these metabolites in urine is lower than the concentration of QD itself. The urinary elimination of unchanged drug is highly pH-dependent with largest excretion at acidic pH. Quinidinone, 3-OH-QD and also hydroquinidine (H-QD, which

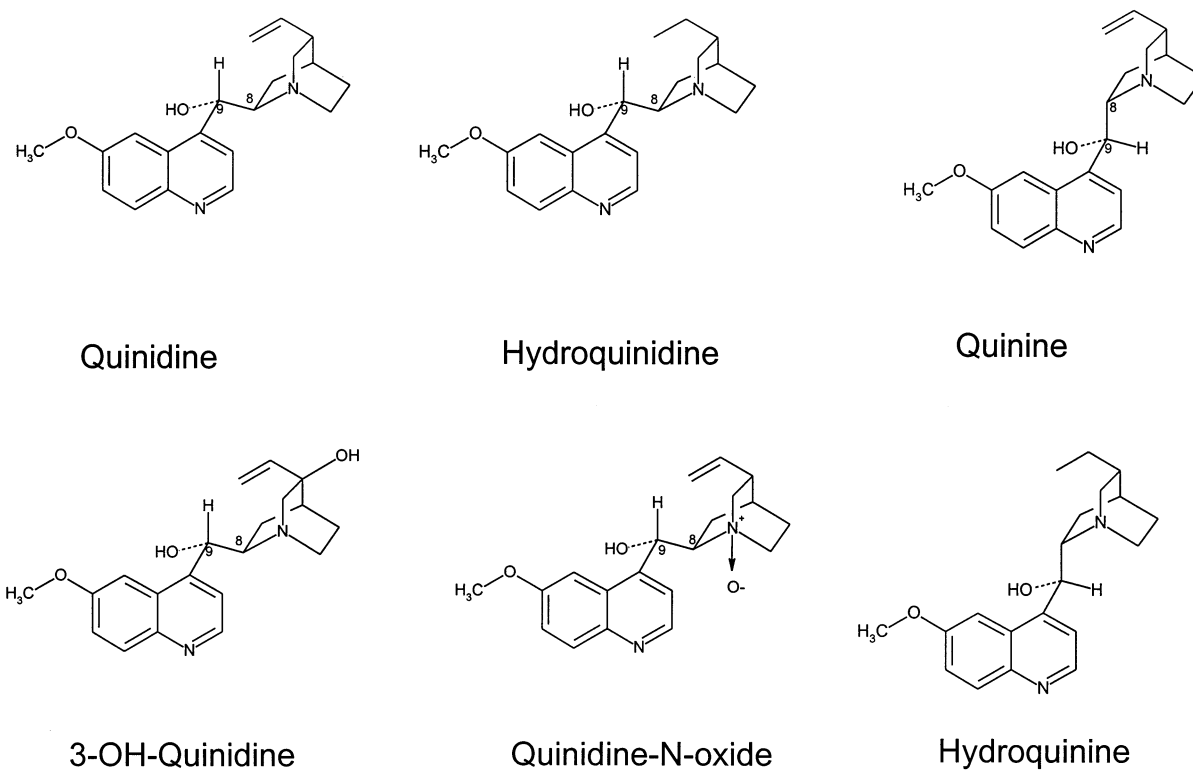

Fig. 1. Chemical structures of quinine, quinidine and related compounds.

is a common impurity in commercial preparations) have equivalent antiarrhythmic potency as the mother compound [1,2]. The therapeutic plasma concentration range is 2–6 μg/ml and for ≥6 μg/ml there is a concentration dependent drug toxicity. The plasma half life is reported to be 4–12 h (mean: 7 h). Plasma protein binding is 75–90%. The structures of QD, H-QD, 3-OH-QD, QD-*N*-OX, QN and H-QN are presented in Fig. 1. It is interesting to note that QD and QN were found to inhibit CYP2D isoenzymes in an opposite way. The human isoenzyme CYP2D6 is known to be virtual absent in certain humans. These persons react as poor metabolizer phenotypes (PMs) in regard to drugs which are almost exclusively metabolized by this isoenzyme (e.g. debrisoquine [3]). QD inhibits this isoenzyme, which converts extensive metabolizer phenotypes (EMs) into PMs for a certain time. QN however, has no effect on the human CYP2D6 isoenzyme. The analogue isoenzyme in rat is CYP2D1 which is inhibited by QN and not by QD [4].

The therapeutic ranges of QD and QN are narrow and there is considerable intersubject variation in the plasma levels of these drugs [1,5]. Thus, monitoring drug levels in body fluids is important for optimization of pharmacotherapy and assessment of patient compliance. Furthermore, it was also found to be justifiable to include QN in drug screening of patients who deliberately harmed themselves and were admitted to the emergency care unit [6] and in investigations of drug related deaths [7]. As QN is present in quinine waters (30–100 mg/l [2], 40–85 mg/l [8]) and thus often used without control for the TDM laboratory, it may influence the results of QD monitoring. Furthermore, some metabolites and the always present hydroforms show pharmacological activity. Thus, it is important to separate these substances, particularly for pharmacokinetic [9–16] and pharmacogenomic [4,9,17] studies. Many different methods for analysis of the compounds of interest in body fluids have been developed, including those based upon fluorimetry [18], immunochemical reactions [18–22], HPLC with UV or fluorescence detection [9–15,23–27] and GC-MS [16]. Commercial immunoassays for TDM of QD in plasma and serum are currently available from various manufacturers.

Recently, capillary electrophoresis (CE) was shown to be an attractive alternative to liquid and gas chromatography for determining drugs and metabolites in body fluids [28–30]. A literature survey revealed very few CE papers about QD and QN. Reijenga et al. developed a capillary isotachophoresis method for analysis of QN in beverages, urine and pharmaceuticals [8], Caslavska et al. reported the determination of QD in urine and serum by micellar electrokinetic capillary chromatography (MECC) with fluorescence detection [31], Steinmann and Thormann described an MECC-based immunoassay for QD in serum [32] and Trenerry and Ward reported an MECC assay for measuring QN in bitter drinks [33]. Furthermore, QN was used as internal standard in a CE-based assay for procainamide in serum [34], as chiral additive in non-aqueous CE [35,36] or as buffer additive for indirect absorption [37] and indirect fluorescence [38] detection in CE with aqueous buffers. No paper was found discussing: (i) the simultaneous CE separation of QN, QD, their hydroderivatives and their major metabolites; and (ii) a comprehensive assay for analysis of these substances in body fluids.

The purpose of this paper was to elucidate the possibilities of employing CE with UV absorption and laser induced fluorescence (LIF) detection for the separation, discrimination and determination of the two diastereomers (QN, QD), their hydroderivatives and two major QD metabolites (3-OH-QD and QD-*N*-OX) in body fluids. Three approaches were used, namely capillary zone electrophoresis (CZE) with β-cyclodextrin (β-CD) in the running buffer, MECC with an organic solvent buffer additive and a competitive binding fluorescence immunoassay for quinidine in which the free tracer and the tracer–antibody complex are separated by CZE or MECC.

2. Experimental

2.1. Chemicals, immunoassay reagents and origin of samples

All chemicals used were of analytical or research grade. QN sulfate dihydrate, β-cyclodex-

trin (β-CD) and sodium fluorescein were purchased from Fluka (Buchs, Switzerland) and QD sulfate dihydrate was from the pharmacy Blüemlisalp (Thun, Switzerland). 3-OH-QD and QD-*N*-OX were a kind gift of Dr Markus Wenk (Kantonsspital Basel, Basel, Switzerland). Stock solutions (about 1 mg/ml) of QN, QD, QD metabolites and fluorescein were made in water. The TDxFLx FPIA reagent kit for drug monitoring of QD in serum or plasma (No. 9506-60) was purchased from Abbott Laboratories (Baar, Switzerland). The reagent pack comprises separate vials for antibody containing solution (solution S; comprising $< 1\%$ goat antiserum) and QD fluorescein tracer solution (solution T; comprising $< 0.01\%$ QD fluorescein tracer in buffer) whose concentrations are not exactly disclosed [39]. Calibrator sera were from Abbott (No. 9506-01; six levels between 0 and 8 μg/ml QD) and calibrator urines were made by spiking blank urine with QD in the same concentration range as for serum.

Urines and plasma samples containing QD stemmed from a controlled clinical study during which healthy volunteers obtained 50 mg QD sulfate 2 h prior to 60 mg dihydrocodeine [40,41]. Samples analyzed were from one volunteer and comprised plasma that was drawn 0.5 and 3.5 h after QD administration (referred to as samples P1 and P2, respectively, in the remainder of this paper) and urine that was collected just prior to and 0–12 h after dihydrocodeine intake (samples U1 and U2, respectively). Furthermore, sera and plasma of patients under QD treatment were collected in the departmental routine drug assay laboratory where they were received for therapeutic drug monitoring by FPIA on the TDxFLx apparatus (Abbott). The FPIA assay was executed according to the manufacturer's instructions. Body fluids containing QN were obtained after self-administration of 5 dl quinine water (Schweppes, whose QN content was determined to be 50 mg/l using the CZE assay as described below). Serum, urine and saliva were collected after 80, 90 and 95 min, respectively. Blank urine, serum and saliva were collected from the same person. All samples were stored frozen at -20°C until analysis.

2.2. Instrumentation and running conditions for MECC and CZE

All MECC measurements were made on the P/ACE System 5510 instrument (Beckman, Fullerton, CA) with a 50 μm I.D. capillary (Polymicro Technologies, Phoenix, AZ, USA) of 27 cm length (20 cm to detector) and UV absorption detection (214 nm) or LIF detection. For LIF, a 325 nm HeCd Laser (Model 4230NB, Liconix, Santa Clara, CA, USA) was used. Emission was measured at 366 nm (band pass filter). If not stated otherwise, the running buffer contained 75 mM SDS, 6 mM $Na_2B_4O_7$, 10 mM Na_2HPO_4 (pH 9.4) and 7% (v/v) 2-propanol. The voltage applied was 20 kV (current: 80 μA). The injection time interval was 4 s (pressure: 0.5 psi). Data were evaluated using the P/ACE Station Software (Beckman). No sample pretreatment was necessary for the MECC experiments, as the proteins in the samples are solubilized by dodecyl sulfate from the running buffer.

CZE measurements were made on the same instrument using a 75 μm I.D. fused-silica capillary (Polymicro Technologies) of 47 cm (40 cm to detector) length and UV absorption detection (214 nm) or LIF detection with the 325 nm HeCd Laser (Liconix) and an emission filter of 450 nm. A constant voltage of 7 kV (current about 21 μA) was applied. The capillary temperature was kept at 20°C and the sample carousel was at room temperature. Pressure injection was effected at 4 s (0.5 psi). If not stated otherwise, the running buffer was composed of 50 mM H_3PO_4 (adjusted with NaOH to pH 2.5) and 15 mM β-CD (added freshly every day). Samples were pretreated by liquid/liquid extraction. Hundred microliters urine, serum or saliva, 10 μl of 2 M Na_2CO_3 solution (pH 11.4) and 300 μl ethylacetate were combined, vortexed for 10 s and centrifuged for 30 s. Two-hundred and forty microliters of the organic (upper) phase was transferred to a glass test tube and evaporated to dryness at 37°C under a gentle nitrogen stream. The residue was redissolved in 100 μl of 10-fold diluted running buffer without CD. Extraction recoveries for QD and QN (500 ng/ml, each, in urine) were determined to be 87.1 and 93.1%, respectively. Similar values were obtained for fortified serum.

2.3. Instrumentation and running conditions for electrokinetic capillary based immunoassays

The P/ACE system 5510 instrument (Beckman) was used. It was equipped with a 50 μm I.D. capillary (Polymicro Technologies) with a total length of 27 cm and effective length of 20 cm. Sample injection occurred via application of pressure (0.5 psi) for 2 s. Solute detection was effected with the LIF detector assembly (Beckman) that was powered by a 488 nm air cooled Argon ion laser (Ion Laser Technology, Salt Lake City, UT, USA) and was equipped with a 488 nm notch filter and a 520 nm band pass filter. Two buffers, an MECC buffer containing 75 mM sodium dodecyl sulfate (SDS), 6 mM $Na_2B_4O_7$ and 10 mM Na_2HPO_4 (pH 9.4) and a CZE buffer composed of 50 mM $Na_2B_4O_7$ (pH 9.5) were used. Voltages applied were 8 (31 μA) and 12 kV (70 μA), respectively. The capillary temperature was held at 20°C and the carousel was at room temperature. In the morning the capillary was rinsed with 0.1 N NaOH, water and running buffer (10 min each). Between runs, wash cycles with 0.1 M NaOH (2 min), water (2 min) and running buffer (4 min) were applied.

Sample preparation comprised the mixing of 25 μl of tracer solution, 25 μl of antiserum solution, 5 μl of an aqueous fluorescein (0.88 μg/ml) solution (internal standard) and 5 μl of serum or urine and incubation for 10 min at room temperature. The mixture was then transferred to the sample vial and injected immediately. Data were evaluated using the P/ACE Station Software (Beckman). Peak heights of the free tracer and internal standard were employed. Calibration graphs were evaluated on a PC using SigmaPlot Scientific graphing software version 2.01 (Jandel, San Rafael, CA, USA). The calibration graphs were constructed by non-linear regression analysis based upon a four-parameter log/logit-model according to [32]

$$y = a + b/\{1 + \exp[-(c + d \times \ln x)]\}, \quad (1)$$

where x is the solute concentration, y the peak height ratio and a, b, c and d the parameters to be determined by regression analysis.

3. Results and discussion

3.1. Separation and detection of quinine, quinidine, hydroquinine, hydroquinidine and two major quinidine metabolites by MECC and CZE

First, the separability of the compounds was assessed with UV detection (214 nm). Selected electropherograms are shown in Fig. 2. Under typical alkaline MECC conditions with dodecyl sufate micelles, QD, QN and their hydroderivatives were found to be inseparable (bottom graph of panel A of Fig. 2). With addition of 5% methanol QD and QN could not be resolved, whereas 5% 2-propanol provided four peaks with almost complete baseline resolution. The two major peaks correspond to QD and QN whereas the small peaks represent their hydroderivatives (Fig. 1, impurities of the standards). Complete separation was obtained via further increase of the 2-propanol content (top graph in panel 2A whose data were gathered with 10% 2-propanol). These experiments show that in absence of a modifier the difference in the solute micelle interaction between QD and QN is none or too small to provide separation. However, the addition of an organic solvent, especially 2-propanol, was found to differentially change the partitioning equilibria which resulted in complete resolution of the two diastereomers and their hydroimpurities (marked as H-QD and -QN in Fig. 2A). Furthermore, attempts in using β-CD (15 or 30 mM) as selector for QN and QD instead of the organic solvent modifier did not reveal any separation of QN and QD (data not shown). Under CZE conditions employing phosphate buffers, QD and QN were determined to comigrate at acidic, neutral and alkaline pH (graphs a, c and e, respectively, in panel B of Fig. 2). These compounds are cations at all three pH values investigated (second pK_a values for QD and QN are 8.8 and 8.5 [1], respectively). With addition of 15 mM β-CD, QD and QN and their hydroderivatives became resolved at all three pH values investigated (graphs b, d and f, respectively, in panel B of Fig. 2). At pH 8, QD was detected prior to QN and the two compounds were barely separated. At neutral and acidic pH, QN was found to migrate faster than QD and

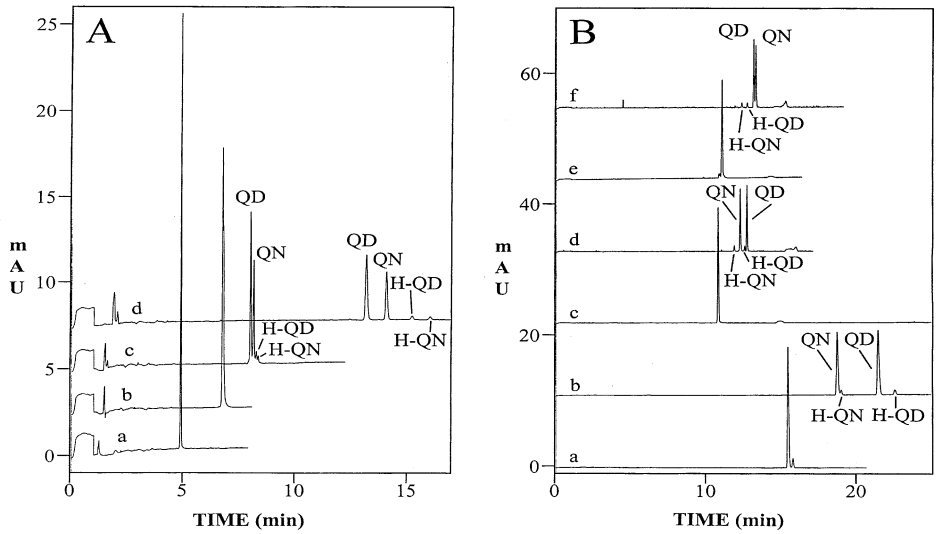

Fig. 2. MECC (panel A, 25 μg/ml each in water) and CZE (panel B, 10 μg/ml each in water)) electropherograms for QN and QD obtained with UV absorption detection at 214 nm. MECC buffers comprised: (a) no additive; (b) 5% (v/v) methanol; (c) 5% (v/v) isopropanol; and (d) 10% (v/v) isopropanol. CZE buffer pH's were (a, b) 2.5, (c, d) 7.0 and (e, f) 8.0 and the buffers comprised (a, c, e) no additive and (b, d, f) 15 mM β-CD. H-QD and -QN refer to hydroquinidine and hydroquinine, respectively. Data are depicted with *y*-axis offsets of: (A) 2.5; and (B) 11 mAU.

separation capacity increased as the pH was decreased. In contrast to data obtained by MECC, these experiments show that β-CD is acting as selector between the two diastereomers QD and QN in absence of the micelles. Without differential complexation, however, the two compounds could not be separated.

The separation of two major QD metabolites (QD-*N*-OX and 3-OH-QD, Fig. 1) from QD and QN was examined by both methods using LIF detection. Typical MECC and CZE data are presented in Fig. 3A–B, respectively. In presence of the micelles and 7% (v/v) 2-propanol, QD-*N*-OX and 3-OH-QD were determined to elute much earlier than QD and were nicely resolved (Fig. 3A). The graph in Fig. 3B presents CZE data obtained with QD, QN (including their impurities), 3-OH-QD and QD-*N*-OX (including an unknown fluorescing impurity marked with an asterisk in Fig. 3A–B, possibly also the hydroderivative) at pH 2.5 with 15 mM β-CD. Under these conditions, 3-OH-QD was detected after QD whereas QD-*N*-OX migrated with a velocity between those of QN and QD. Data comparison revealed quicker separations in the alkaline MECC format. In CZE, the total run time is rather high. For sufficient separation of the hydroanalogue of QN, conditions leading to shorter run times could not be used. From a separation point of view, both buffers were found to be suitable.

For optimization of detection sensitivity, LIF responses were evaluated with emission filters of 479, 450, 405 and 366 nm. For MECC in presence of 7% (v/v) 2-propanol, the 366 nm filter provided the largest responses (data not shown), whereas for CZE at pH 2.5, the 450 nm filter was found to provide highest sensitivity (Fig. 4). With the use of the four emission filters, no significant differences in the fluorescence responses of QD and QN were observed. In any case, LIF detection with gathering of wavelength resolved solute emission [42] would be the best approach as it would provide highest assay sensitivity and the possibility of solute identification via comparison of emission spectra. Commercial instrumentation featuring such a detector, however, is not available. Furthermore, measuring

QN in absence of micelles and otherwise identical conditions as used for MECC, provided a similar response as in MECC. LIF responses at 366, 405, 450 and 479 nm were determined to be about 76.5, 143.2, 98.6 and 82% compared to those observed in MECC. Thus, the fluorescence wavelength with highest response at alkaline pH (shift towards lower wavelengths), but not the magnitude of the response, appears to be influenced by the presence of the micelles. However, comparison of the two electropherograms registered with the same model mixture by MECC at alkaline pH and CZE at low pH (Fig. 3) reveals that the sensitivity of the two methods is quite different. The MECC assay (366 nm filter) is characterized with about 17-fold smaller responses compared to those observed by CZE at pH 2.5 (450 nm filter). This difference is mainly due to the pH dependent fluorescing properties of QN and QD. Furthermore, LIF solute detection was found to provide much higher (> 20-fold) sensitivity compared to that obtained with UV detection at 214 nm (see below). Thus, for analysis of QD and QN in body fluids by CE, major focus was geared towards the use of LIF detection.

3.2. Analysis of body fluids by MECC and CZE

For MECC, a 50 μm I.D. capillary was employed and body fluids were directly injected and detected by LIF with an emission filter of 366 nm. Selected data obtained by MECC with direct urine injection are presented in Fig. 5. All substances of interest are shown to be detectable free of endogenous urinary compounds (first and second electropherograms from bottom). Data obtained with real world samples are presented in Fig. 6. Analysis of the urine U1 provided electropherograms in which QD (12.6 μg/ml), its major metabolites and possibly also H-QD could be detected (graph a and inset of Fig. 6A). In a urine collected 90 min after self-administration of 25 mg QN, QN and most likely also its hydroderivative were monitored (graph b of Fig. 6A). It was interesting to realize that the resolution between the two QD metabolites observed after urine injection was higher compared to that seen after application of standards only (see data of Fig. 4). To prove that this is due to a matrix effect, the two metabolites were analyzed in 10, 50 and 90% diluted urine (data not shown) and resolution was

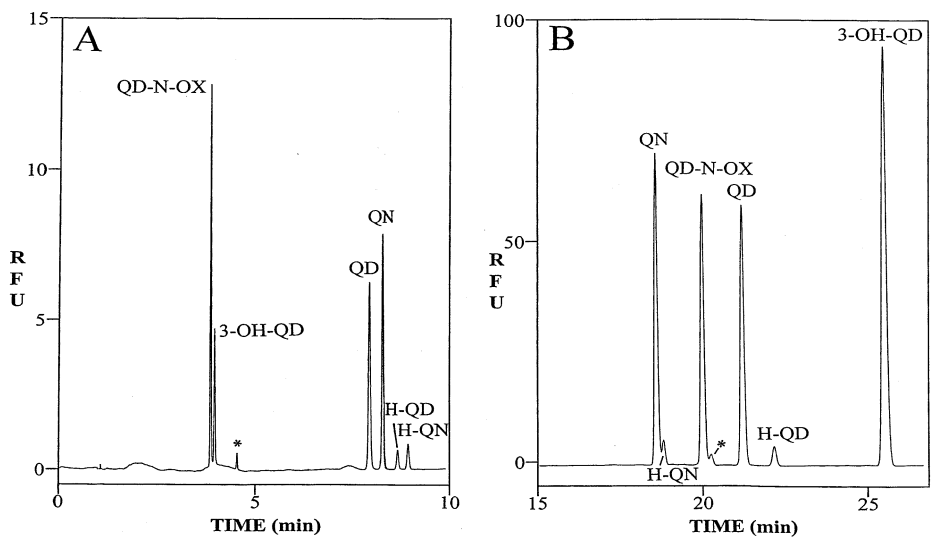

Fig. 3. Electropherograms obtained with an aqueous standard solution containing QD (2.5 μg/ml), QN (2.5 μg/ml), QD-*N*-OX (5 μg/ml) and 3-OH-QD (5 μg/ml) and analysis by: (A) MECC with 7% (v/v) 2-propanol as buffer additive; and (B) CZE at pH 2.5 in presence of 15 mM β-CD. LIF detection was effected at: (A) 366; and (B) 450 nm. The peaks marked with an asterisk refer to an impurity originating from the QD-*N*-OX standard. Other conditions as described in Section 2.2.

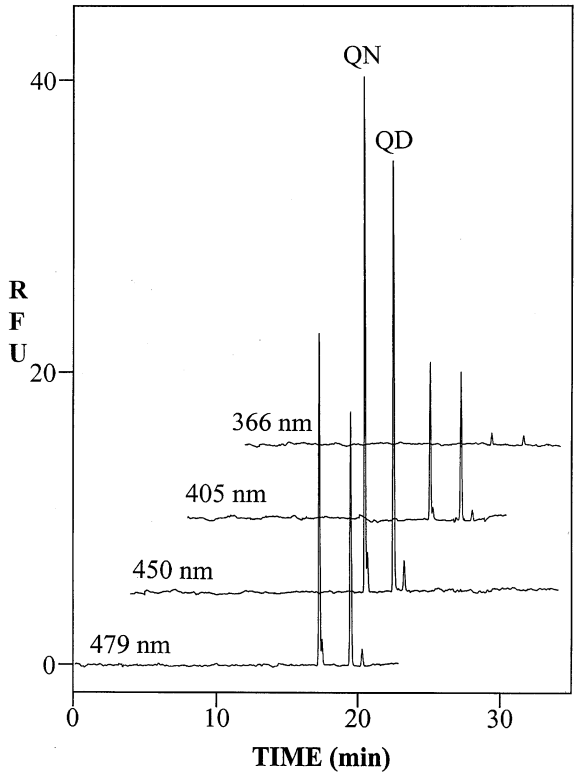

Fig. 4. CZE electropherograms with LIF detection for QN and QD (sample: 1 μg/ml each dissolved in water) and monitored at different emission wavelengths. For presentation purposes, data are depicted with x- and y-axis offsets. Other conditions as for Fig. 3B.

determined to be 5.3, 4.7 and 3.0, respectively. The data presented in panel B of Fig. 6 represent typical electropherograms for analysis of plasma and serum samples by MECC with direct sample injection. QD (concentrations of 152 and 51 ng/ml for plasma samples P1 and P2, respectively) and QD-*N*-OX could be determined in the two plasma samples collected after administration of 50 mg QD. 3-OH-QD was not detected in these samples. Furthermore, analysis of the serum collected 80 min after intake of 25 mg QN revealed the presence of QN at a concentration of 227 ng/ml. QD and QN drug levels were estimated by four-level external calibration using drug concentrations between 20 and 200 ng/ml and salicylate (1.96 μg/ml) as IS. Calibration graphs were found to be linear with $r > 0.994$ and the detection limits for QD and QN were determined to be about 10 ng/ml. QD metabolites were not quantitated.

With UV detection at 214 nm, assay sensitivity was found to be much lower. This is documented with the MECC data of undiluted urine U1 presented in Fig. 7. In the case of UV detection, QD (12.6 μg/ml) was monitored as small peak (panel A) and the two QD metabolites could not be detected (inset of panel A). The opposite is illustrated with LIF detection (panel B). It is important to realize that the two experiments were performed with different amounts of isopropanol in the buffer. The higher amount present in the case of electropherogram Fig. 7A lead to higher

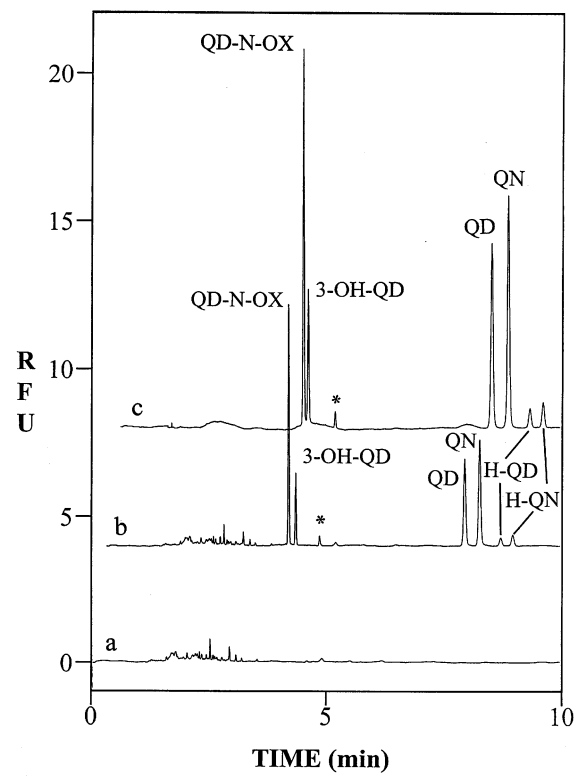

Fig. 5. MECC electropherograms with direct sample injection and LIF detection of: (a) urine blank; (b) urine blank fortified with QD (1.25 μg/ml), QN (1.25 μg/ml), QD-*N*-OX (2.5 μg/ml) and 3-OH-QD (2.5 μg/ml); and (c) an aqueous standard solution containing QD (2.5 μg/ml), QN (2.5 μg/ml), QD-*N*-OX (5 μg/ml) and 3-OH-QD (5 μg/ml). Data are depicted with x- and y-axis offsets of 0.6 min and 4.0 RFU, respectively. The peaks marked with an asterisk refer to an impurity of QD-*N*-OX. Other conditions as for Fig. 3A.

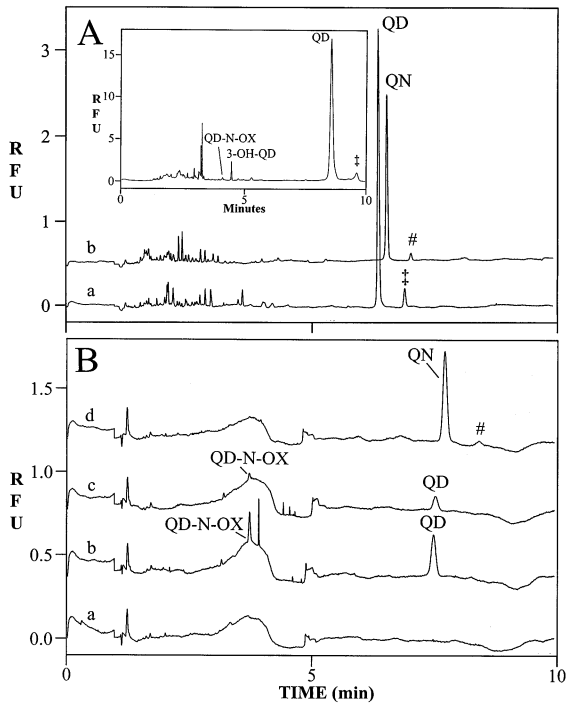

Fig. 6. MECC electropherograms with LIF detection and direct sample injection of: (A) urines; and (B) plasma and serum specimen. Data presented as graphs a and b in panel A stemmed from the 10-fold diluted urine U1 and from the undiluted QN containing urine, respectively. Data obtained with undiluted urine U1 are presented as inset. The graphs a–d of panel B were those obtained with blank serum, samples P2, P1 and the QN containing serum, respectively. Data are depicted with y-axis offsets of: (A) 0.5; and (B) 0.4 RFU. Peaks marked with # and ‡ are likely those produced by H-QN and -QD, respectively. Experimental conditions as for Fig. 3A.

resolution which is in agreement with the data presented in Fig. 2A. Furthermore, in both cases, QD was found to be incompletely separated from another compound that produced a small peak only. Application of the urine containing QN (1.2 μg/ml, see also data with LIF detection presented as graph b of Fig. 6A) to UV detection, QN was barely recognized as a tiny peak but could not be quantitated (data not shown). Furthermore, QD could not be detected in the plasma samples P1 and P2 and QN was not seen in the serum and saliva samples containing QN. Under the employed conditions, the UV absorption detection mode does not permit quantitation of sub μg/ml concentrations of QD and QN. The MECC data with direct sample injection indicate, however, that UV detection could be used for cases that require a ppm sensitivity, namely therapeutic drug monitoring and clinical toxicology.

Data obtained with urinary extracts that were analyzed by CZE in a 75 μm ID capillary and LIF detection at 450 nm are presented in Fig. 8. As above for direct injection of body fluids under MECC conditions, the extracts could be analyzed without interferences (compare electropherograms a–c of Fig. 8). For the samples collected after intake of QD, QD and its metabolites could be monitored. This is illustrated with the data presented in Fig. 9A. As was the case with MECC (see above), no 3-OH-QD was detected in the two plasma samples. The QD concentration in the three samples was determined to be 0.15, 0.06 and 12.6 μg/ml, respectively. Electropherograms registered with the saliva, serum and urine samples containing QN are presented in panel B of Fig. 9. In all three cases, QN could easily be determined, the concentrations being 0.1, 0.3 and 1.2 μg/ml, respectively. QD and QN drug levels were estimated by single level calibration using a urine calibrator containing 500 ng/ml of each drug. The detection limit was estimated to be at 10 ng/ml. It is important to realize that with the extraction procedure employed the solutes of interest were somewhat diluted. Assay sensitivity could be enhanced by concentration of the solutes during extraction and/or electroinjection from a sample prepared in a very low conductivity matrix [43]. Employing a different CE instrument featuring a 50 μm I.D. capillary and UV detection at 210 nm, QD and QN concentrations ≤ 0.1 μg/ml could not be determined (data not shown). Thus, QD and metabolites in the two plasma samples were not recognized and QN in saliva could be seen barely only. Drug levels that were determined by four-level external calibration using drug concentrations between 100–1000 ng/ml (calibration graphs were linear with $r^2 > 0.9732$), were found to compare well with those obtained by CZE and LIF detection.

3.3. Analysis of body fluids by a CE-based immunoassay for quinidine

Abbotts's QD FPIA reagents were employed for immunological recognition and analysis in CE using both MECC (as described previously [32]) and CZE conditions at alkaline pH. For the two CE-based immunoassay formats, calibration curves were measured (Fig. 10). For MECC (Fig. 10A), the serum samples of Abbott's calibration kit were analyzed whereas for CZE (Fig. 10B), fortified blank urines were employed as calibrators. In the MECC electropherograms (panel A) the first peak represents the internal standard (IS, fluorescein, 74 ng/ml) and the second peak the free QD tracer (FT). The tracer–antibody complex is only barely visible and it was found to almost coelute with the IS. In the CZE electropherograms (panel B) the first peak represents the antibody–tracer complex (C), the second peak is the free tracer FT and the third peak is the IS (same as for MECC). The peak height of the free tracer is a measure of reactivity. As expected, the peak height of the free QD tracer was found to increase as the QD concentration in the sample was increased whereas the magnitude of the tracer–antibody complex decreased (Fig. 10B, not well seen in Fig. 10A). It is important to note that, during the gathering of the CZE data the instrument did not inject very reproducibly. Thus, the peak heights varied strongly. Peak height ratios, however, were reproducible. The calibration graphs for both methods are presented as insets. For MECC, a nice calibration curve comparable to that measured previously [32] was obtained. In the case of CZE, however, an almost rectangular shaped graph was obtained, which indicates a change in the immunochemical equilibrium in absence of the micelles. For a better measuring range, the relative amounts of the reagents should be changed (not yet investigated). Nevertheless, the calibration graphs were found to permit an estimation of the QD content in body fluids. The open circles (plasma samples and urines for the cases of MECC and CZE, respectively) in both curves represent the samples from the DHC study. The relative height values are compared with the estimated QD concentration of the CZE measurements with LIF detection (see above). The open squares in the MECC calibration curve

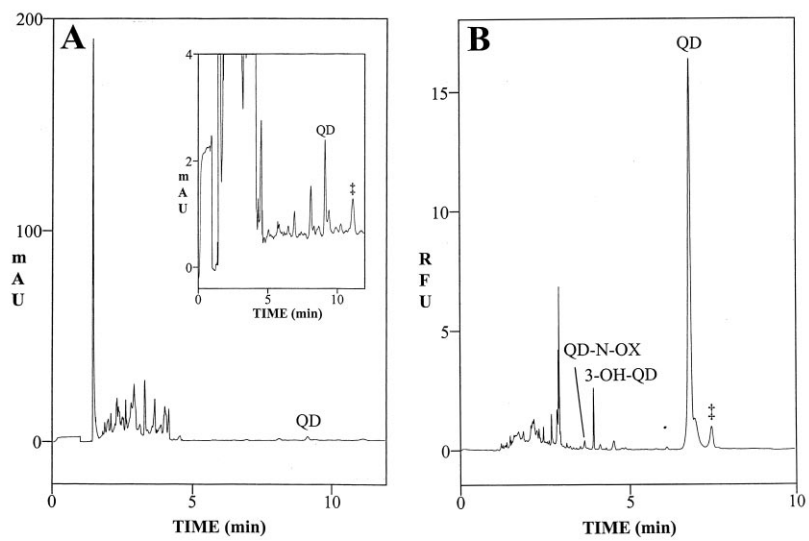

Fig. 7. MECC electropherograms with: (A) UV absorption detection at 214 nm; and (B) LIF detection at 366 nm of directly injected urine sample U1. All experimental conditions are the same as for Fig. 3A except that the buffer for panel A contained 10% instead of 7% (v/v) isopropanol. The currents were about 66 and 80 μA, respectively. The peak marked with ‡ is assumed to be that of H-QD.

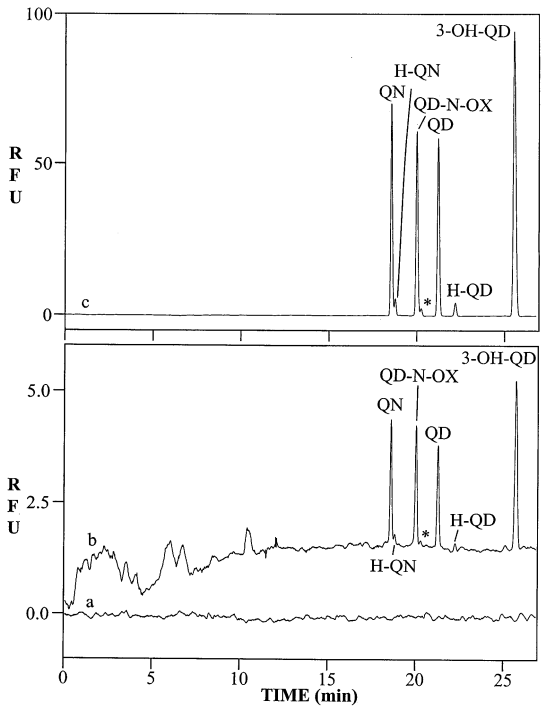

Fig. 8. CZE electropherograms with LIF detection of: (a) an extract of urine blank; (b) an extract of urine blank fortified with QD (125 ng/ml), QN (125 ng/ml), QD-*N*-OX (250 ng/ml) and 3-OH-QD (250 ng/ml); and (c) an aqueous standard solution containing QD (2.5 μg/ml), QN (2.5 μg/ml), QD-*N*-OX (5 μg/ml) and 3-OH-QD (5 μg/ml). The peaks marked with an asterisk refer to an impurity of QD-*N*-OX. The *y*-axis offset between graphs a and b is 1.5 RFU. Other conditions as for Fig. 3B.

are the relative height values for four patient plasma samples compared with the QD data determined with FPIA.

The two CE-based immunoassay formats were employed to assess the recognition of QD, QN and the two QD metabolites by the antibody against QD. Serum and urine samples were analyzed at alkaline pH by MECC and CZE, respectively. The data presented in Fig. 11 are those obtained for the analysis of blank body fluids and blank body fluids fortified with 1 μg/ml QN, 3-OH-QD, QD-*N*-OX or QD (from bottom to top). As discussed above, the peak height of the free tracer is a measure of reactivity. It is clearly visible that QD gives the highest response in both formats and the QD metabolites react with a crossreactivity of 20–30%. QN at the 1 μg/ml level provides responses that are comparable to those of blank serum (left panel) and blank urine (right panel). The same was found to be true for samples containing 0.1 and 10 μg/ml QN (data not shown). It can be concluded that the antibody used is recognizing QD and its metabolites and FPIA data for QD are thus not reflecting the QD levels only. This is in agreement with the data given by the manufacturer of the FPIA kit [39] and elsewhere [19]. The same antiserum was also reported to be responsive to H-QD [20]. Due to lack of standards, this crossreactivity was not studied by the electrokinetic capillary immunoassays. Furthermore, it was interesting to find that the antibody is not at all reacting with QN, the diastereomer of QD. Discrimination between QD and QN by this antibody appears to be excellent and QD can be quantified in presence of QN, i.e. in samples of patients who consumed quinine water prior to blood or urine collection. This is further illustrated with the MECC immunoassay data presented in Fig. 12. The electropherograms presented in Fig. 12A were obtained with a urine blank (bottom graph), the DHC study urines containing about 12.6 and 3.7 μg/ml QD, respectively, and the urine with about 1.2 μg/ml QN that was collected after intake of quinine water (top graph). MECC based serum data are presented in panel B. In presence of QN, no elevated tracer peaks were noted. However, all body fluids containing QD provided electropherograms with increased tracer peaks.

The QD sensitivity of Abbott's FPIA kit is reported to be 0.2 μg/ml [39]. As is documented with the data presented in Fig. 10, calibrator B (QD concentration: 0.5 μg/ml) was found to provide a higher tracer signal than calibrator A (0.0 μg/ml). Furthermore, data obtained with the two DHC study plasma samples that contain about 50 and 150 ng/ml QD (see above) indicate that the limit of detection in the capillary format is actually somewhat better (see data in Fig. 12B). For MECC analysis of a serum containing 1 μg/ml QD, RSD values for detection times and relative heights of FT were determined to be 0.7 and 10.3%, respectively ($n = 4$).

4. Conclusions

Although the two diastereomers QD and QN have slightly different physical properties, they were found to be inseparable by CE based on their differences in protolysis. In MECC with dodecyl sulfate micelles and alkaline pH, sufficient differential partitioning was observed in presence of small amounts of 2-propanol. Addition of β-CD instead of the organic modifier, did not reveal any separation of the two diastereomers. Using CZE, however, β-CD was found to permit the separation of these compounds over a wide pH range. Best discrimination was observed at low pH. MECC with 7% 2-propanol and CZE at pH 2.5 with 15 mM β-CD in the background electrolyte were determined to be ideal media for separation of QD, QN, their hydroderivatives and the two QD metabolites 3-OH-QD and QD-*N*-OX. Using MECC, all body fluids can directly be injected whereas in the case of CZE, proteinaceous samples have to be cleaned up. MECC (fluorescence detected at 366 nm) and CZE at pH 2.5 (fluorescence detected at 450 nm) were determined to provide a ppb sensitivity, the detection limits for QD and QN with direct injection of a body fluid being about 10 ng/ml each. This ppb detection sensitivity was found to permit the analysis of QD in urine and plasma, both major QD metabolites in urine and QD-*N*-OX in plasma after ingestion of 50 mg QD sulfate, and QN in serum, saliva and urine samples that were collected after intake of 25 mg QN. Thus, CE with LIF detection permits the elucidation of pharmacokinetic and drug metabolism properties with a sensitivity that is typically achieved in assays based upon micellar liquid chromatography with direct sample injection and fluorescence detection [24], and based upon HPLC with solute extraction and fluorescence detection [9] or UV absorbance detection [12,13,23,26]. Compared to HPLC, advantages of using CE-based assays are small consumption of chemicals and buffers, inexpensive separation capillaries and direct application of untreated body fluids as samples. The rather high operational cost of the HeCd laser is a disadvantage. For therapeutic drug monitoring and clinical toxicology of QD (therapeutic range: 2–5 μg/ml) and QN (range: 3–7 μg/ml), CE assays with UV detection provide sufficient sensitivity. Further-

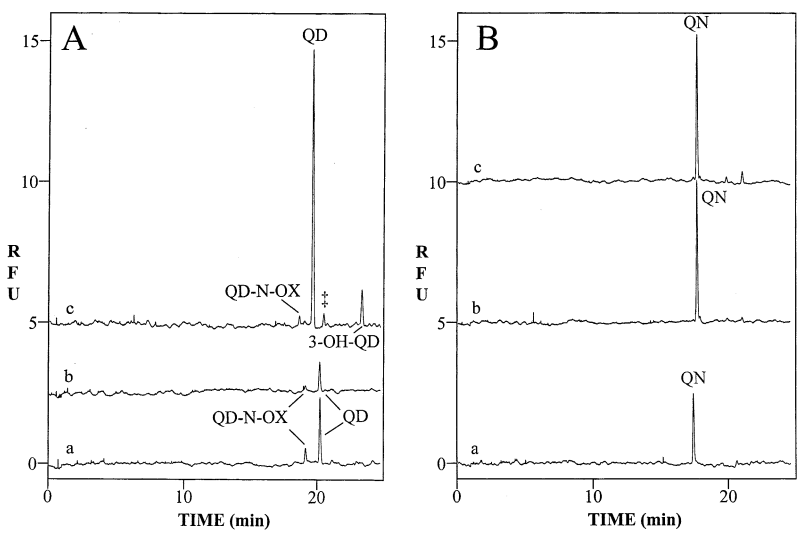

Fig. 9. CZE electropherograms with LIF detection of extracts of: (A) QD; and (B) QN containing samples. Data presented as graphs a–c in panel A stemmed from samples P2, P1 and U1 (extract of U1 was 20-fold diluted with 10-fold diluted running buffer), respectively, whereas the graphs a–c of panel B were those obtained with saliva, serum and urine (urinary extract was five-fold diluted with 10-fold diluted running buffer), respectively. The peak marked with ‡ is likely that produced by H-QD. Data are depicted with *y*-axis offsets of: (A) 2.5; and (B) 5.0 RFU. Experimental conditions as for Fig. 3B.

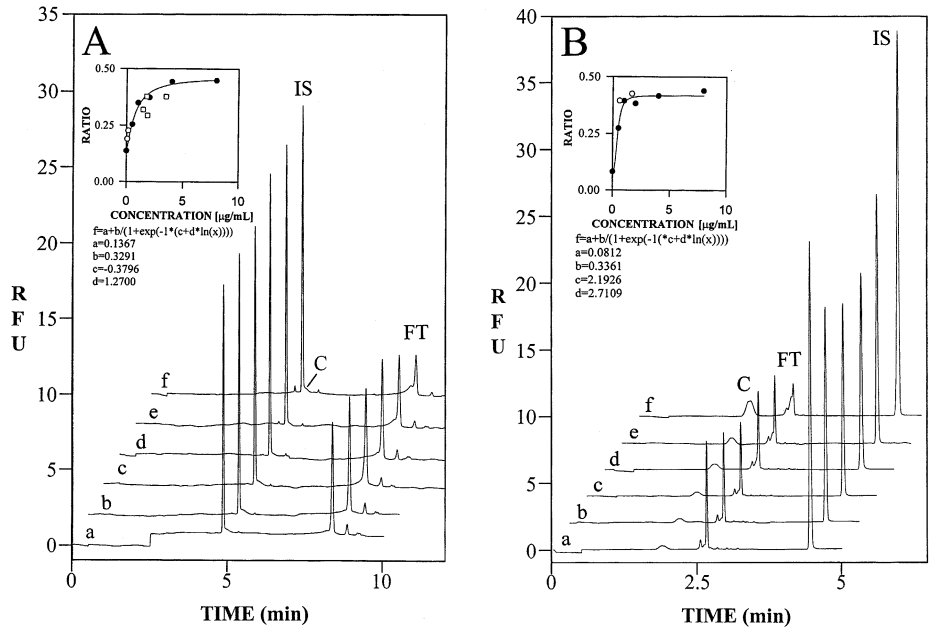

Fig. 10. Immunoassay calibration data obtained via: (A) MECC of the six Abbott serum calibrators; and (B) CZE of corresponding homemade urine calibrators. Insets depict the constructed calibration graphs with the filled circles as calibration data points. Data of samples containing 0, 0.5, 1.0, 2.0, 4.0 and 8.0 μg/ml QD are presented as graphs f–a, respectively. Peaks representing the free tracer, the antibody–tracer complex and the internal standard are marked with FT, C and IS, respectively. Data are presented with x- and y-axis offsets of: (A) 0.5 min and 2.0 RFU, respectively; and (B) 0.3 min and 2.0 RFU, respectively. For further explanations refer to text. Experimental conditions as described in Section 2.3.

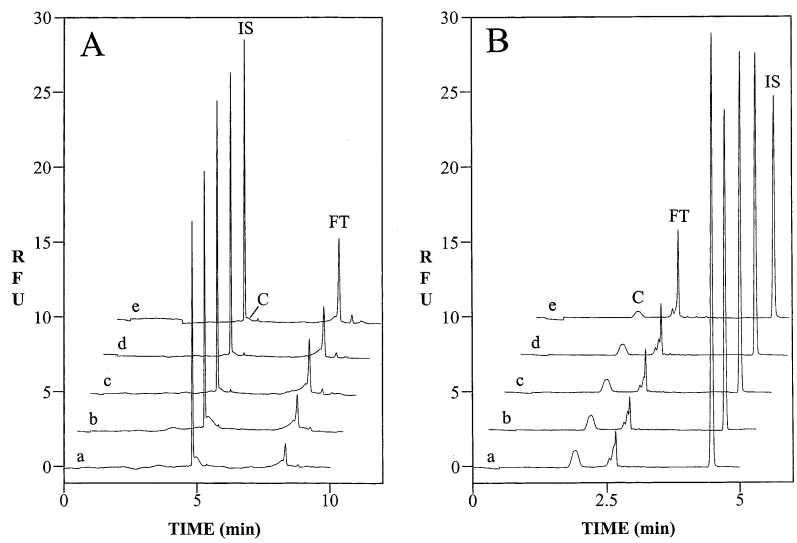

Fig. 11. Immunoassay data obtained via: (A) MECC of blank and fortified test sera; and (B) CZE of blank and fortified test urines. Graphs a–e represent data monitored with blank body fluid, and blank body fluid fortified with QN, 3-OH-QD, QD-*N*-OX and QD (1 μg/ml each), respectively. For further explanations refer to text. Data are presented with x- and y-axis offsets of: (A) 0.4 min and 2.5 RFU, respectively; and (B) 0.3 min and 2.5 RFU, respectively. Experimental conditions as described in Section 2.3.

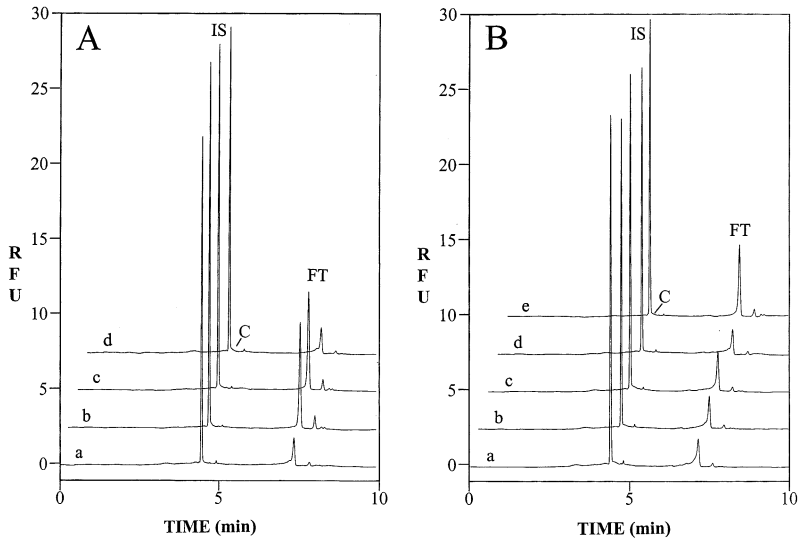

Fig. 12. MECC immunoassay data of: (A) urines; and (B) plasma and serum specimens that were obtained from volunteers and patients. Urine samples analyzed comprised: (a) blank urine; (b) urine U1; (c) urine U2; and (d) the urine containing QN that was collected 90 min after drinking 0.5 l Schweppes. Data depicted in panel B were obtained with: (a) blank serum; (b) plasma sample P1; (c) plasma sample P2; (d) serum collected 80 min after drinking Schweppes; and (e) patient sample containing 1.9 μg/ml QD (value determined by FPIA). Data are presented with x- and y-axis offsets of 0.3 min and 2.5 RFU, respectively.

more, MECC- and CZE-based immunoassay formats are shown to permit rapid screening of body fluids for the presence of QD and analogs on the lower ppb concentration levels. The FPIA antiserum against QD employed in this study, however, was determined not to crossreact with QN. This stereospecificity is in agreement with that very recently reported using the TDx and AxSYM analyzers [44] and is similar to that described for antibodies that were geared towards the recognition of QN and the insensitivity to QD [22].

Acknowledgements

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