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Article

2D Capillary Electrophoresis Hyphenated with Spectral Detection for the Determination of Quinine in Human Urine

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The possibilities of a column coupling two-dimensional capillary electrophoresis (2D CE) combined with fiber-based diode array detection (DAD) for the direct, highly reliable and ultrasensitive quantitative determination of quinine in real multicomponent ionic matrices (human urine) are demonstrated in this work. The capillary isotachophoresis (CITP) stage provided an on-line sample pretreatment (elimination of interfering matrix constituents, preseparation and preconcentration of the analyte) before the capillary zone electrophoresis (CZE) separation. Due to the large volume (30 μL) sample injection and CITP sample preconcentration, a simple absorbance photometric detection was sufficient for obtaining very low concentration limits of detection (∼8.6 ng/mL). The combination of the different separation mechanisms (CITP and CZE) resulted in enhanced separation selectivity. This enabled us to obtain a pure analyte zone in the directly injected real samples suitable for qualitative and quantitative evaluation. The spectral DAD allowed (i) characterization of the purity (i.e., spectral homogeneity) of the analyte zone; and (ii) preliminary indication of structurally related compounds (i.e., potential biodegradation products of quinine), via characteristic spectra recorded in intervals of 200–800 nm. The CITP–CZE–DAD method was characterized by favorable performance parameters that are suitable for its routine biomedical use. One of the primary benefits of the CITP–CZE–DAD method is the possibility of performing direct injections of real biological samples while avoiding external sample preparation procedures and, therefore, enhancing the reliability and applicability of analyses and the potential for method automatization and miniaturization.

Introduction

Quinine (QUI) is a natural alkaloid with antipyretic (fever-reducing), antimalarial, analgesic (painkilling) and anti-inflammatory properties. Still, it is often recommended as a relief for cramps. Resistance of the parasite *Plasmodium falciparum* to quinine appears to be significantly less than with chloroquine, and thus QUI is still widely used (1). In addition to the use of QUI in the treatment of chloroquine-resistant strains of *Plasmodium falciparum*, it is used in patients with severe and complicated malaria (2). In humans, QUI is oxidized to several more polar hydroxy metabolites, e.g., 3-hydroxyquinine and 2′-hydroxyquinine (3–5). Bannon *et al.* identified ten QUI metabolites in urine using a gas chromatography–mass spectrometry (GC–MS) technique (5). Plasma protein binding is 70–90%. Furthermore, commercial preparations of QUI contain up to 10% of the hydroform of QUI (hydroquinine) (3, 4). Because of the previously mentioned biological activities of QUI, this drug must be monitored in biological systems.

Several capillary electrophoresis (CE) techniques [capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC) and capillary isotachophoresis (CITP)] and detection techniques [ultraviolet (UV) and laser-induced fluorescence (LIF)] have been used for the separation and determination of QUI in real matrices such as beverages, pharmaceuticals, urine and plasma (2, 6–9). Reijenga *et al.* (6) developed a CITP separation method with UV detection and liquid–liquid extraction for the determination of QUI in urine. The concentration limit of detection (LOD) for QUI was 5,000 ng/mL. Tsimachidis *et al.* (7) applied CZE separation with UV detection and solid-phase extraction for the determination of QUI in urine samples. The concentration LOD for QUI was 180 ng/mL. Intra-day and inter-day reproducibility of the QUI determination ranged in intervals of 1.9–4.9%. Zhang *et al.* (8) applied CZE separation and UV detection for blood and urine samples that were pretreated before the analyses by solid-phase extraction. In pretreated blood and urine samples, the concentration LODs for QUI were 30 and 20 ng/mL, respectively. The recoveries were in the intervals of 84.65–92.35% and 82.53–90.63% for urine and blood samples, respectively. Corresponding precision data were 4.70 and 4.91% for urine and blood samples, respectively. Zaugg and Thormann (2) developed CZE and MEKC separation methods with LIF detection for the sensitive determination of QUI in biological samples (plasma, serum and urine). They obtained a 10 ng/mL concentration LOD of QUI, with (CZE–LIF) or without (MEKC–LIF) liquid–liquid extraction pretreatment of the samples. Altria and Simpson (9) used high-voltage CZE for the analysis of QUI in pharmaceuticals. They obtained a 3,000 ng/mL concentration LOD of QUI with fluorescence detection and an analysis precision of 1.5% relative standard deviation (RSD). In all cases in which QUI was analyzed by CZE or CITP in biological material, an external sample preparation was necessary.

CITP coupled on-line with CZE provides a very significant CE tool applicable for trace analytes and direct injections of unpretreated complex ionic matrices (10). To our best knowledge, however, no CE–CE method for the separation of QUI in complex biological matrices has been used thus far. In many cases, spectral diode array detection (DAD) has appeared to be a simple solution in the preliminary characterization of electropherograms in the z-axis (absorbance versus migration time versus wave length, x-y-z); for example, in the analysis of drugs in body fluids (11). In this way, DAD is expected to enhance the reliability of results reflected in the validation parameters (12). However, no CE–DAD method for the spectral evaluation of QUI in biological matrices has been used thus far.

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The aim of the present work was to develop a hyphenated CITP−CZE−DAD method for (i) a highly sensitive and selective determination of trace QUI; (ii) direct injections of unpretreated biological samples; and (iii) preliminary indication of the structurally related compounds of QUI (potential biodegradation products) present in real biological matrices (human urine). The developed method should be useful for routine use in biomedical and clinical laboratories.

Experimental

Instrumentation

A capillary electrophoresis analyzer EA-102 (Villa-Labeco; Spišská Nová Ves, Slovakia), assembled in the column-coupling configuration of the separation unit, was used in this work for performing the CITP−CZE runs. Electrode compartments with connecting channels to the separation compartment that were hydrodynamically (membrane) closed (Villa-Labeco) were employed. The samples were injected by a 30-µL internal sample loop of the injection valve of the analyzer. An isotachophoretic module was provided with an 800 µm i.d. fused silica capillary tube of 90-mm length and a contactless conductivity detector. The CZE column was the same as the CITP column, except it included 300 µm i.d. and 140-mm length.

A multiwavelength photometric absorbance DAD Smartline PDA Detector 2800 (Knauer; Germany) was connected to an on-column photometric detection cell, mounted on the CZE column via optical fibers. The detector operated under the following conditions: (i) scanned wavelength range 200−800 nm; (ii) integration time 6 ms; (iii) scan interval 0.2 s; (iv) number of accumulations 1.

Before use, the capillaries were not treated by any rinsing procedures to suppress an electroosmotic flow (EOF). A dynamic coating of the capillary wall by means of methylhydroxyethylcellulose (MHEC 30,000; Serva; Heidelberg, Germany) present in leading and background electrolyte solutions served this purpose (13). The separating electrolytes in the capillaries were replaced by fresh ones between each run. CITP and CZE analyses were carried out in the cationic regime of the separation (i.e., cathodic movement of the analytes) with direct injections of the samples. The experiments were performed in constant current mode at 20°C. The driving currents applied were 300 µA (CITP) and 120 µA (CZE).

Data evaluation and performance parameters

Performance parameters of the method were evaluated according to the International Conference on Harmonization (ICH) guidelines (12). The peak area of QUI was corrected for the migration time (14). The photometric detector was set at a 330-nm detection wavelength when evaluating the performance parameters. The parameters of calibration line for QUI were calculated by using QCExpert version 2.5 statistical software (Trilobyte; Prague, Czech Republic).

Processing and comparing DAD spectra

The migration and spectral data were acquired and processed by a EuroChrom program (version 3.05, Knauer).

Background correction (subtraction of background spectrum from the raw spectrum of the analyte) (15) was performed to minimize the impact of the electrolyte system on the QUI spectrum. The corrected spectrum was further smoothed by the procedure of Savitzky-Golay (16) (implemented in EuroChrom software) with a 5-point window.

Homogeneity of QUI spectra was expressed via Pearson's correlation coefficients (PCCs) (17). A value of PCC higher than 0.99 is assumed to provide acceptable certainty in confirmation of the identity of the analyte (15), i.e., a match of the tested (QUI in urine) and reference (QUI in demineralized water) spectra.

Chemicals and samples

The electrolyte solutions were prepared from chemicals obtained from Merck (Darmstadt, Germany), Sigma−Aldrich (Steinheim, Germany) and Fluka (Buchs, Switzerland) in water demineralized by a Rowapure-Ultrapure water purification system (Premier; Phoenix, AZ). All chemicals were of analytical grade. The solutions of the electrolytes were filtered before use through disposable membrane filters of 0.8 µm pore size (Millipore, Molsheim, France).

Quinine hydrochloride dihydrate was obtained as a reference substance from Sigma. The beverage (bitter drink), Kinley, was obtained from a local store. The content of QUI in the beverage was determined in this work by the method proposed therein.

Procedures for sample and standard solution preparations

The stock solution of QUI reference substance (i.e., standard stock solution) was prepared by dissolving 10 mg of the powder in 10 mL of demineralized water with 0.5 mmol/L H_2SO_4 and 1 mmol/L NaH_2PO_4. Working standard solutions were made by an appropriate dilution of the standard stock solution with the mixture consisting of 0.5 mmol/L H_2SO_4 and 1 mmol/L NaH_2PO_4 in demineralized water.

Blank, model (spiked) and real (after QUI administration) urine solutions were also made in 0.5 mmol/lLH_2SO_4 and 1 mmol/L NaH_2PO_4 background, and after an appropriate dilution (10 times), they were directly injected. Each sample was kept in the freezer (−18°C) until use.

A 300-mL volume of the bitter drink (Kinley), with a 38.45 mg/L determined concentration of QUI in the beverage, was administered orally to a healthy young female volunteer. The total content of QUI in this dose was 11.535 mg. The real urine sample was taken 2.5 h after QUI was administered. The total volume of this urine was 650 mL. The real urine sample was frozen (−18°C) immediately after sampling and kept in the freezer until use. The sample was thawed out just before manipulation and preparation of the sample (as mentioned previously) and immediately injected into the sampling loop of the electrophoretic analyzer.

Results and Discussion

CITP−CZE separation conditions

The working conditions such as buffer constituents, concentration of leading, terminating, and carrier cations, pH, and driving

currents were optimized for the CITP–CZE separations performed in the column coupling arrangement of the hydrodynamically closed system with enhanced sample load capacity. Therefore, the compatibility of the CITP and CZE electrolytes and minimization of dispersion effects (electroosmotic and termal) were included among the most important tasks in method optimization. Here, the maximal match between terminating and carrier electrolyte, low conductivity buffers and EOF suppressing buffer additive were applied. Additionally, the working conditions reflected the following:

(i) Properties of the analyte such as mobility (position of the analyte between leading and terminating cations in the CITP stage, mobility match of the analyte and carrier cation in the CZE) and tendency to adsorb because of less polar fluorescing moiety (phosphates and polymers as competitive adsorbing additives).
(ii) Accompanied sample matrices (preseparation selectivity of CITP versuss fine selectivity of CZE, electronic removing of major matrix constituents in the CITP step), because of multicomponent ionic urine matrices.
(iii) Very low concentration levels of the injected analyte (large volume sample injection and consecutive CITP preconcentration). Employing the competitive adsorbing additive (NaH_2PO_4) and ionization additive (H_2SO_4) to the samples was prerequisite for a reproducible handling and separating of the trace (ng/mL) concentration levels of QUI.

The major matrix constituents (MAJ) were selectively removed in the CITP stage (Figure 1A), preventing an overload of the narrower CZE capillary and eliminating potential interferences. The minor matrix constituents (MIN) were not removed in the CITP stage, although it is also possible with this system, because the CZE separation system provided sufficient separation selectivity, allowing the complete separation of QUI from MIN (Figure 1B).

The optimized electrolyte systems, considering high separation efficiency and separation selectivity, low concentration LOD, short analysis time, and minimum sample handling, are the following: (i) CITP electrolytes, leading electrolyte (LE), 10 mmol/L sodium acetate; 20 mmol/L acetic acid; 1 mmol/L NaH_2PO_4; 0.1% (w/v) MHEC; pH 4.5; terminating electrolyte (TE), 10 mmol/L β-alanine (BALA); 10 mmol/L acetic acid; pH 4.3; (ii) CZE carrier electrolyte, 25 mmol/L BALA; 25 mmol/L acetic acid; 0.1% (w/v) MHEC; pH 4.3.

Electropherograms from the CITP–CZE separations of QUI present in a model urine sample at the concentration close to its LOD recorded under optimized separation conditions are shown in Figure 1. In addition to the possibility of performing effective separations of trace (ng/ml) QUI in urine samples with a direct injection, i.e., without an external sample pretreatment (Figures 1A and 1B), the UV-VIS spectra of QUI in a 200–800 nm scanned wavelength range can be directly recorded during the separation process (Figure 1C). The spectrum of QUI in spiked urine was compared with the QUI spectrum recorded in demineralized water and a 0.9994 value of PCC indicated an excellent match of both spectra, i.e., confirmation of a sufficient purity of the QUI zone in the spiked urine sample. This result confirmed an excellent separation selectivity produced by the proposed CITP–CZE method. Then the approved spectrum in Figure 1C was used as a reference spectrum for the real urine samples evaluated in a following section.

Performance parameters of the CITP–CZE–DAD method

The CITP–CZE–DAD method with optimized separation parameters was validated using the model urine samples. All resulting statistical data and performance parameters of the method are given in Table I.

The increased dimensionality of separations due to the on-line coupled CITP and CZE techniques was favorable for (i)

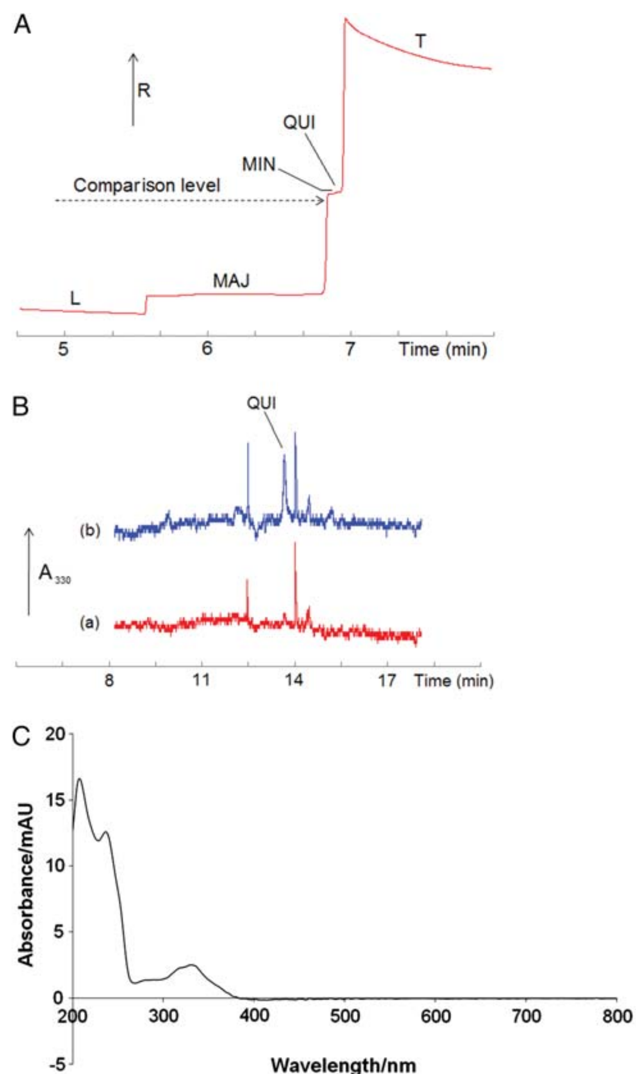

Figure 1. Direct determination of QUI in model urine sample by means of the CITP–CZE–DAD method. Electropherograms were obtained in isotachophoretic (A) and zone electrophoretic (B) stages of on-line coupled CITP–CZE runs. The processed UV-VIS spectrum of QUI present in spiked urine (C) served as a reference spectrum. Electrolyte systems are given in the "CITP–CZE separation conditions" section. Other working conditions and the processing procedure of the spectra are given in the "Experimental" section. The urine samples were diluted 10 times. Blank urine (lower trace in Figure 1B) served as a matrix reference and the same urine spiked with ~10 ng/mL concentration of QUI (upper trace in Figure 1B) served as a model QUI sample. Comparison level indicates a voltage value starting the column switching process.

the separation efficiency (N) and height equivalent to one theoretical plate (H); and (ii) sample loadability and, consecutively, sensitivity. The obtained LOD values [signal-to-noise (S/N) = 3] and limit of quantification (LOQ; S/N = 10) clearly favor the use of this hyphenated method in the ultra-trace quantitative determination of QUI in urine matrices, even if a conventional absorbance photometric detection is used. As discussed previously, the LODs for QUI in biological (mostly urine) samples, achieved using CE with UV detection and off-line sample pretreatment, ranged in intervals of 20–5,000 ng/mL (6–8). Moreover, using the proposed CITP–CZE–DAD (UV) method, the achieved LOD of QUI (8.6 ng/mL) is lower than that achieved by CE with LIF or fluorescence detection (10–3,000 ng/mL) (2, 9).

The determination coefficient (r^2) reflected good linearity in a 20–200 ng/mL concentration range of the standard QUI solution. The recovery values, based on a ratio of the QUI peak areas obtained in different matrices (urine and water) at three different concentrations (30, 90 and 180 ng/mL), were between approximately 92 and 95%. This indicated a slight effect of the urine matrix on the analyte signal. Regardless, it still indicates acceptable accuracy of the proposed CITP–CZE–DAD method.

The evaluated repeatability is acceptable, which is clearly visible from the values of RSDs of (i) migration time of QUI (RSD_t); (ii) intercept (RSD_a) of the calibration line of QUI; (iii) slope (RSD_b) of the calibration line of QUI; (iv) series of the recovery measurements (RSD_{Rec}).

The deliberate altering of the operational parameters, i.e., concentration of leading (9.5–10.5 mmol/L) and carrier (24–26 mmol/L) cations, pH ($\pm$0.1), in the robustness test applied for the urine samples resulted in fluctuations of t_m that were less than approximately 3.5% of the values obtained under the standard conditions. This favorable robustness of the method highlighted the feasibility of its routine use.

Table I
Performance Parameters of the CITP–CZE–DAD Method and Parameters of Calibration Lines of QUI Measured with Spiked Urine Samples*

Parameter	Value/range
t_m (min)†	5.89
s_{tm} (min)	0.0538
RSD_{tm} (%), $n = 6$	0.91
a (mV)	0.1315
s_a (mAU)	0.0026
RSD_a (%), $n = 6$	1.98
b (mAU/ng/mL)	0.1661
s_b (mAU/ng/mL)	0.0028
RSD_b (%), $n = 6$	1.69
r^2	0.9986
LOD (ng/mL)	8.62
LOQ (ng/mL)	28.74
N†	26,628
H (μm)‡	5.26
Recovery (%)	91.94–94.23
RSD_{Rec} (%), $n = 6$	2.36–3.86
Robustness (t_m) (%)	<3.49

*Note: Separation conditions are described in the "CITP–CZE separation conditions" section; the other working conditions are described in the "Experimental" section. The samples were urine diluted 10 times. The concentration of QUI was 50 ng/mL unless otherwise stated.
†CZE analysis time.
‡CZE capillary length.

Application in analysis of biological samples

The proposed and proved CITP–CZE–DAD method was applied in bioanalysis in which the concentration levels of trace QUI were monitored in human urine taken after the QUI administration (details were described previously). The content of QUI in the bitter drink administered orally was determined by the same CITP–CZE–DAD method. The migration position of QUI in the electropherograms was confirmed by spiking the real samples with the QUI standard. A representative electropherogram, shown in Figure 2A, illustrates the CZE profile of the human urine with the biologically excluded QUI. Here, the on-line CITP sample pretreatment was prerequisite for achieving the excellent separation selectivity and sensitivity when separating the real biological samples in the CZE stage.

It was found in this study that the concentration of the unchanged QUI, excluded 2.5 h after the administration of a 11.535-mg dose of QUI, was 1.2252 μg/mL. It was calculated as the average value from the six consecutive experiments (an illustrative electropherogram is in Figure 2A). The RSD value of this determination was 2.37%, which confirmed the high precision of the method. The experimentally obtained concentration of the excluded QUI represented a 796.38 μg mass of QUI excluded in a 650-mL urine volume. A percentual amount of the unchanged QUI excluded 2.5 h after the administration of the dose of QUI was 6.90%. This updates the published

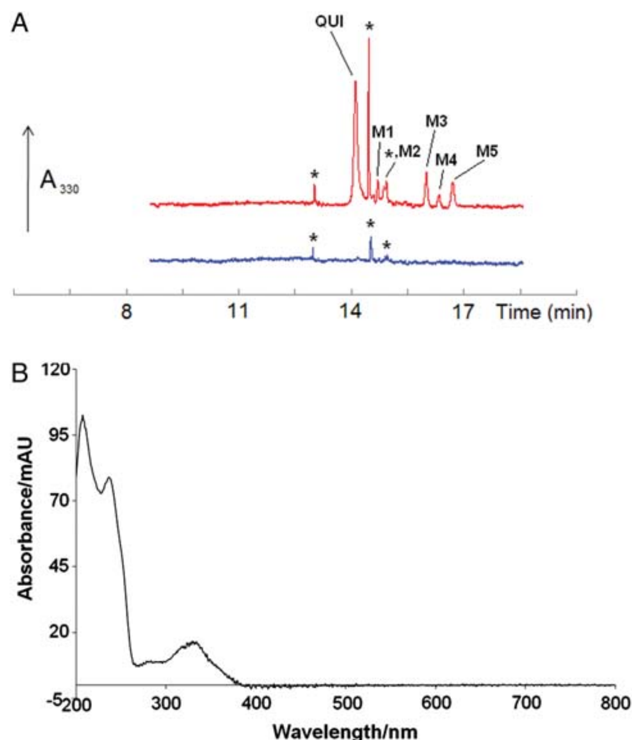

Figure 2. Electropherogram from the CZE step of the CITP–CZE–DAD combination, illustrating the electrophoretic profile of real urine taken 2.5 h after oral QUI administration (a 11.535-mg dose) to a healthy female volunteer (A); corresponding processed spectrum of QUI (B). The urine sample was diluted 10 times. The concentration of QUI in the sample was found to be 122.52 ng/mL. Blank urine (lower trace in Figure 2A) served as a matrix reference. Separation and other working conditions are the same as in Figure 1. Sample preparation is described in the "Experimental" section. Asterisks indicate sample matrix constituents.

pharmacokinetics of QUI, in which the maximum blood level is obtained in 1–3 h after oral administration and approximately 20% of the drug is excreted renally in unchanged form or as conjugates (18).

The reliability of the quantitative determination of QUI in human urine was demonstrated by the spectral evaluation of the QUI peak. The DAD spectrum, shown in Figure 2B, was examined to prove the purity (i.e., spectral homogeneity) of the QUI zone. This proof was based on a required match between the reference and tested QUI spectra, and differences between the tested and interfering spectra. This investigation was motivated by a high probability of interferences in a multicomponent matrix (human urine), and especially, an increased risk of a contamination of such matrix by the structurally related homologues of the analyte (i.e., human urine obtained from a real biological process of QUI biodegradation). The processed spectra provided relevant spectral information for QUI present in both the model sample (reference spectrum of QUI in the spiked urine sample in Figure 1C) and the real sample (tested spectrum of QUI in the real urine sample in Figure 2B). Homogeneity of the tested QUI spectrum, obtained from the real urine sample and six consecutive measurements, was expressed via PCC and accompanying RSD value (Table II). Here, the average matching factor was considerably higher than 0.99. This indicated, with a very high probability, the pure analyte zone (without any interfering compound), i.e., sufficient selectivity of the CITP–CZE separation and the high reliability of the quantitative determination of QUI in the urine samples taken after QUI biodegradation.

Table II
Spectral Evaluation of Electrophoretic Zones Obtained from the Urine Samples by the CITP–CZE–DAD Method*

Parameter	Spiked QUI	Real QUI	M1	M2	M3	M4	M5
PCC	0.9997	0.9993	0.8749	0.9503	0.9680	0.9754	0.9826
RSD (%), $n = 6$	2.24	1.97	2.79	2.89	2.17	2.37	1.86

*Note: Separation conditions are described in the "CITP–CZE separation conditions" section; the other working conditions and the processing procedure of the spectra are described in the "Experimental" section. The urine samples were diluted 10 times and directly injected into the analyzer.

To demonstrate other analytical possibilities of the proposed method, the spectral characterization was also applied for unknown zones (M1–M5) that appeared in the CZE profiles of the urine samples taken after QUI biodegradation (Figure 2A). Because we do not have any standard of the QUI structural homologues, the reference QUI spectrum (Figure 1C) was compared with the M1–M5 spectra (Figure 3) and the results were expressed via corresponding PCC values. The PCC values of the processed spectra of M1–M5 in the urine samples taken after the QUI biodegradation ranged in the interval of 0.87–0.98 (Table II). These relatively high PCC values can still indicate a structural relationship, especially of M2–M5

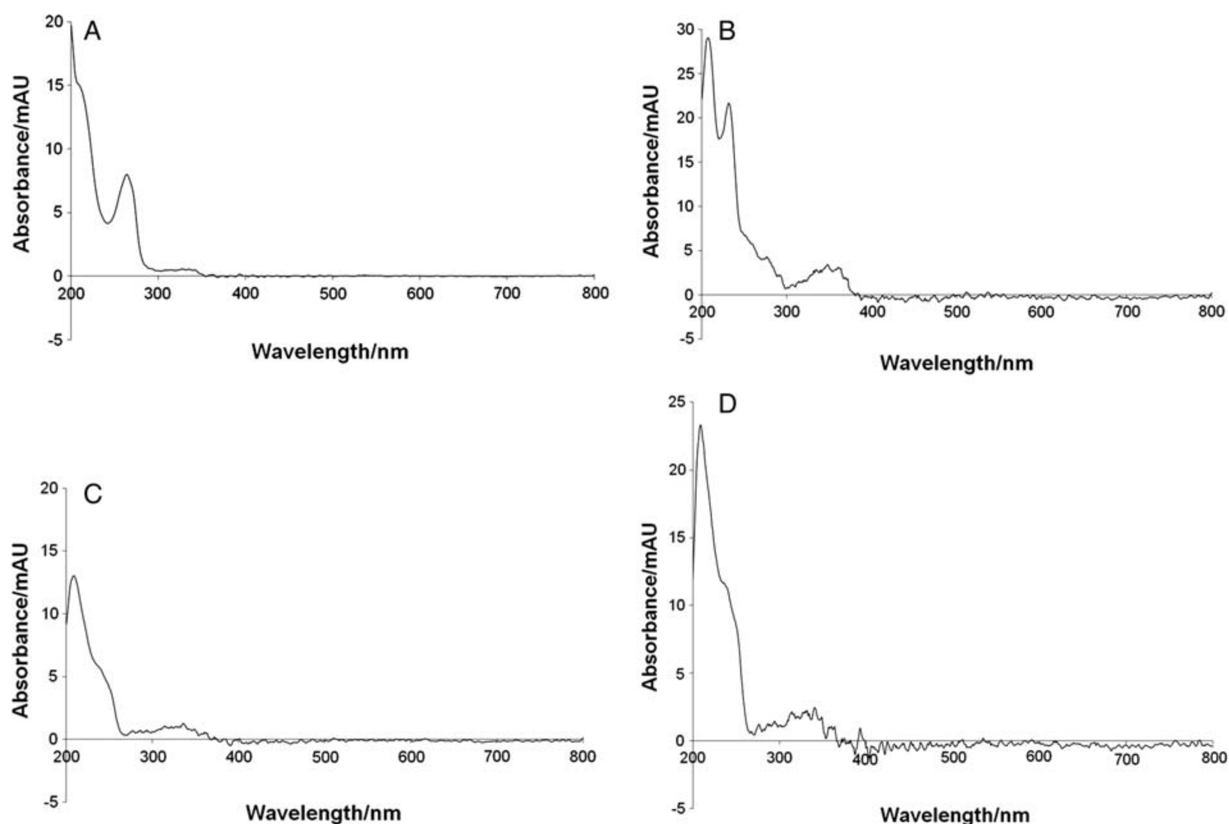

Figure 3. Processed spectra of potential QUI metabolites tested with PCCs values higher than 0.90 (M2–M5 in Figure 2A): M2(A); M3(B); M4(C); M5(D). All materials and conditions are the same as in Figure 2.

and QUI (PCC > 0.95). From this, one can assume that M2–M5 could be structurally related biodegradation products (metabolites) of QUI. Among them, more polar ionizable metabolites with higher molecular weight (e.g., 3-hydroxy-quinine; 2'-hydroxyquinine; 2'-quinone; 10,11-dihydroxydihydroquinine and quinine-N-oxide) can be expected for M2–M5 (3–5). Nevertheless, more sophisticated spectral methods (e.g., mass spectrometry or nuclear magnetic resonance) should be used for the definite confirmation of this preliminary assumption. On the other hand, the DAD spectra can still be a pragmatic solution in preliminary characterization of the on-line pretreated real biological profiles, because of their low cost and minimum requirements (e.g., composition of separation electrolytes).

Conclusion

It was concluded that this analytical approach based on on-line combined CE techniques, namely CITP and CZE, is highly effective for the sample preparation, selective separation and sensitive determination of biologically active compounds in real biological matrices in one run. The integrated on-capillary spectral DAD detection provides additional valuable analytical information in the separated electrophoretic zones, namely, their spectral homogeneity (revealing mixed zones) and spectral match (revealing basic structural relationship). Such three-dimensional (3D) profiles (migration time versus absorbance versus wavelength) offer a relatively complex view of the analyzed sample that can significantly enhance the reliability of analytical results.

The proposed CITP–CZE–DAD method was approved via the performance parameters that highlighted the feasibility of its routine use. The application example presented in this paper clearly shows the CITP–CZE–DAD method as one of the most complex and superior from among the methods that can be applied for the biomedical analysis of QUI. This hyphenated method can effectively replace the established single column CE methods with UV or even fluorescence detection used for QUI in biological matrices that are less sensitive (CE–UV), more expensive and less versatile (CE–fluorescence) and demand an external sample preparation (both). From this point of view, CITP–CZE–DAD is simple and suitable for automatization and miniaturization, and with promising potential for other molecules and matrices because of its versatility.

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