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Analysis of oxybutynin and *N*-desethyloxybutynin in human urine by dispersive liquid-liquid microextraction (DLLME) and capillary electrophoresis (CE)

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A dispersive liquid-liquid microextraction (DLLME) procedure combined with capillary electrophoresis/diode array detection (CE/DAD) was developed and applied to the determination of oxybutynin (OXY) and (DEO) in human urine. The electrophoretic analyses were performed using a 50 µm i.d. fused silica uncoated capillary with an effective length of 36.5 cm and a 50 mmol L^{-1} solution of triethylamine, pH 3.0, as the background electrolyte. The temperature was set at 30 °C and a constant voltage of +30 kV was applied. Some experimental parameters that affect DLLME's extraction efficiency, such as the type and volume of the extraction and disperser solvents, extraction time and pH were studied and optimized. The optimal DLLME condition was found using the following method: 2.5% (w/v) NaCl was added to 5.0 mL of urine, the pH was adjusted to 11.0 and 140 µL of carbon tetrachloride as the extraction solvent and 260 µL of acetonitrile as the disperser solvent were quickly added. The performance criteria for linearity, sensitivity, precision, accuracy, recovery and stability have been assessed and were within Guidance for Industry: Bioanalytical Method Validation. The mean extraction recoveries for OXY and DEO were 71.4% (SD 6.4%) and 60.9% (SD 8.5%), respectively. The method showed linearity over the concentration range of 90-300 ng mL^{-1} and 187.5-750 ng mL^{-1}, with correlation coefficients of 0.990 and 0.998 for OXY and DEO, respectively. Intra-day and inter-day precision and accuracy assays for these analytes were studied at three concentration levels and were lower than 15%.

1 Introduction

Oxybutynin (OXY) is an antimuscarinic drug that can be used for the treatment of overactive bladder (OAB).[1-3] It is a tertiary amine,[4, 5] which is rapidly absorbed from the gastrointestinal tract[6-9] and extensively metabolized by cytochrome P450 enzymes to *N*-desethyloxybutynin (DEO),[7, 10, 11] the main active metabolite that has plasma concentrations approximately 4-10 times higher than those of the parent drug[11].

OXY and DEO lead to ureter and urinary bladder smooth musculature relaxation, which results in a reduction of both intravesical pressure and urinary bladder contraction frequency.[13] However, their pharmacological activities are not only restricted to the urinary tract. Systemic effects have been reported,[7, 14] such as constipation, dry mouth, confusion, blurred vision and tachycardia, which are the main adverse effects that contribute to treatment non-adherence.[5, 8, 9, 15] The administration of higher doses is related to a lower tolerability to treatment[7] and the use of transdermal[5, 11, 16] or extended-release oral formulations[8] can minimize side effects.

Sample preparation is an extremely important step to obtain sensitive and accurate results;[17] its main purposes are isolation and enrichment of the analytes of interest[18] while maintaining their compatibility with the analytical system to be used.[19] In recent research, efforts have been made in the development of efficient, economical and miniaturized sample preparation techniques.[19, 20] Dispersive liquid-liquid microextraction (DLLME) is a very simple and quick extraction procedure based on the rapid introduction of a suitable combination of an extraction and a disperser solvent into an aqueous sample.[19-21] It is based on the equilibrium distribution of analytes between donor (sample) and acceptor (organic solvent) phases.[17] The advantages of this technique include low cost, simplicity of operation, rapidity, high recovery and high enrichment factor (EF).[17, 20, 22] The disadvantages are that the entire extraction process is manual[21] and the centrifuging step requires the greatest amount of time within the process.[21, 23-26]

For OXY and DEO, few studies involving their analysis in biological matrices have been described and most of them involved plasma and liquid-liquid extraction,[8, 27-29] plasma and solid phase extraction[12, 30] or plasma and precipitation method.[31] The separation techniques used for the quantification of these analytes and, in some cases, of their enantiomers were HPLC,[11, 27] LC-MS/MS[12, 28-30] and GC.[8, 31] Thus, to the best of our knowledge, no method has been reported for the extraction of OXY and DEO from human urine samples using a microextraction technique and no capillary electrophoresis (CE) method is available for their determination. Therefore, in this study, a simple, rapid and accurate CE method for the simultaneous analysis of OXY and DEO in urine using DLLME as the sample preparation technique was developed.

2 Experimental

2.1 Chemicals and reagents

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OXY and DEO were generously supplied by Watson Laboratories (Corona, CA, USA) and Orgamol (Switzerland, France), respectively. Lidocaine (LID), used as the internal standard (IS), was purchased from Sigma Aldrich (St. Louis, MO, USA).

Purified water used for the preparation of the solutions was obtained from a Millipore Milli-Q Plus (Mildford, MA, USA). All reagents used were at least of analytical grade. Dichloromethane (CCl_2H_2), tetrachlorethylene (C_2Cl_4), chlorobenzene (C_6H_5Cl) and sodium chloride were obtained from Merck (Darmstadt, Germany). Carbon tetrachloride (CCl_4) and phosphoric acid were purchased from Synth (São Paulo, SP, Brazil). Ethanol (C_2H_6O), methanol (CH_4O), acetonitrile (C_2H_3N), sodium borate, sodium phosphate, 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris) and triethylamine (TEA) were obtained from JT Baker (Philipsburg, NJ, USA). Acetone (C_3H_6O) and chloroform (CCl_3H) were obtained from Mallinckrodt (Philipsburg, NJ, USA) and sodium hydroxide (NaOH) was purchased from Vetec (Rio de Janeiro, RJ, Brazil). All other chemicals were of at least analytical grade or of the highest purity available.

2.2 Apparatus and analytical conditions

Analyses were performed on an Agilent Technologies CE system (Waldbronn, Germany) model G1600A consisting of an analyzer, an automatic sampler and a diode array detector operating at 204 nm, where the absorption is higher. The CE ChemStation software was used for instrument control, data acquisition and data analysis. An uncoated fused silica capillary (Microsolv, Eatontown, NJ, USA) of 50 µm i.d., with a total length of 45 cm and an effective length of 36.5 cm was used to perform the separations. Before its first use, the capillary was conditioned by rinsing with 1 mol L^{-1} NaOH for 30 min, followed by water for 30 min. Every day prior to and at the end of analyses, it was rinsed with NaOH 0.1 mol L^{-1} for 10 min and water for the same amount of time. Between analyses, rinsing was performed with 0.1 mol L^{-1} NaOH for 2 min, water for 2 min and background electrolyte (BGE) for 3 min. Next, a voltage of 30 kV was applied for 2 min. The electrophoretic separations were carried out in 50 mmol L^{-1} TEA solution (pH adjusted to 3.0 with a 1.5 mol L^{-1} solution of H_3PO_4). The capillary temperature was set at 30°C, a constant voltage of +30 kV was applied and the samples were introduced by hydrodynamic injection (50 mbar for 20 s). All electrolytes were filtered through a 0.45 µm membrane filter and degassed by sonication (Maxiclean model 1450, brand Unique). Employing the established condition of analysis, reproducibility of the migration times and peak areas were evaluated by five consecutive injections of a unique sample using the same BGE. Under these conditions, relative standard deviation (RSD) values for the migration time and peak area of the analytes were 0.6% and 3.3% for OXY and 0.5% and 1.7% for DEO. So, it was decided to change the BGE solution after each 5 analytical runs.

2.3 Preparation of standard solutions and urine samples

Stock standard solutions of OXY and DEO were prepared by dissolving each compound in methanol to obtain a concentration of 1 mg mL^{-1}. Working standard solutions were prepared to provide OXY and DEO concentrations of 50, 25, 15, 10, 5, 2.5 and 1 µg mL^{-1}. For LID, a 10 µg mL^{-1} solution was also prepared in methanol. All of these solutions were stored at -20°C in amber glass tubes and were protected from direct light.

Drug-free urine samples were provided by healthy volunteers that declared no exposure to any drug for at least 3 months and the samples were kept frozen at -20°C before use. To eliminate particulate matter, the frozen urine samples were thawed in a water bath set at 37°C and centrifuged for 10 min at 1092 × *g*. The supernatants were filtered through a 0.45 µm cellulose acetate membrane filter.[32-34] The volunteers gave written informed consent to participate in the investigation, and the research protocol was approved by the Research Ethics Committee of the Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Brazil.

2.4 Optimization of analytical separation conditions

2.4.1 Choice of BGE type

The effect of BGE type on the separation of OXY and DEO was evaluated by performing the electrophoretic separation using 50 mmol L^{-1} sodium phosphate buffer with pH values of 3.0 and 7.0, 50 mmol L^{-1} sodium borate buffer, 50 mmol L^{-1} TEA solution and 50 mmol L^{-1} Tris/H_3PO_4 solution. The last three BGEs had a pH of 3.0. At this step, a fused silica uncoated capillary with 50 µm i.d. and 36.5 cm of effective length, a voltage of +25 kV, temperature of 25 °C and a hydrodynamic injection (50 mbar for 20 s) were used.

2.4.2 Effect of the BGE concentration

The effect of the BGE concentration on the separation of OXY and DEO was examined at concentrations of 15, 25, 50, 75 and 100 mmol L^{-1} using the BGE previously selected at pH 3.0. The other analytical conditions were described in the section 2.4.1.

2.4.3 Effect of capillary temperature on the resolution

To determine the optimum temperature for the resolution of OXY and DEO, electrophoretic runs at different capillary temperatures (10, 20, 30, 40 and 50 °C) were performed. During the evaluation of this parameter, the capillary, voltage and sample injection used before were maintained.

2.4.4 Effect of voltage on the efficiency

The effect of the voltage on resolution, efficiency and migration time of OXY and DEO by CE was evaluated for 20 and 30 kV. The analytes migration time and resolution between peaks were calculated and an optimal voltage for the analysis was determined.

2.5 Dispersive liquid–liquid microextraction procedure

In a 10 mL conical bottom glass tube, an aliquot of 5.0 mL of a human urine sample was spiked with 50 µL of OXY and DEO standard solution (50 µg mL^{-1}) and 25 µL of the IS solution (10 µg mL^{-1}). For the first tests, a mixture containing 400 µL of a disperser solvent and 100 µL of an extraction solvent was injected rapidly using a 1.0 mL syringe into the sample solution (pH adjusted to 10 using NaOH 10 mol L^{-1}). After 2 min, the cloudy solution formed was centrifuged at 2260 × *g* for 5 minutes. Then, the dispersed fine

droplets of the extraction solvent were concentrated in the bottom of the tube and were removed and quantified using a 100 µL micro syringe. The volume of the sediment phase was determined, and 50 µL were transferred to a clean tube and submitted to solvent evaporation. The residue was redissolved in 50 µL of water and further analyzed by CE. All experiments were performed in triplicate and the best condition for the parameter tested was chosen and maintained for the next optimization experiments. The peak area of the analytes was used to evaluate the extraction efficiency.[35] The EF calculation was completed based on the equation presented in Berijani's work.[23]

2.6 Method validation

The validation was performed according to: "Guidance for Industry: Bioanalytical Method Validation".[36]

System suitability was carried out each validation day using ten consecutive runs. To access the selectivity, 6 human urine samples were analyzed to ascertain that no endogenous peak would interfere with OXY and DEO signals under the conditions previously established.

Calibration curves were obtained by spiking aliquots of 5 mL human urine with standard solutions of OXY to obtain urinary concentrations of 90, 120, 150, 180, 240, 270 and 300 ng mL^{-1} and DEO in concentrations of 187.5, 200, 250, 375, 500, 600 and 750 ng mL^{-1}. The IS was used at the final concentration of 50 ng mL^{-1}. OXY/IS and DEO/IS peak area ratios were plotted against the drug concentrations to obtain the respective calibration graph. Regression analysis by the least-squares method was used to calculate the calibration equation and correlation coefficient (r).

The limit of quantification (LOQ) of the method was also evaluated and defined as the lowest concentration that could be determined with accuracy and precision below 20% over six analytical runs using human urine samples (5 mL) spiked with concentrations of 90 ng mL^{-1} for OXY and 187.5 ng mL^{-1} for DEO.

Urine samples at concentrations of 120, 180 and 300 ng mL^{-1} for OXY and 200, 500 and 750 ng mL^{-1} for DEO were used for the precision and accuracy assays. The precision was calculated as RSD (%) within a single day (intra-assay) and between day-to-day (inter-assay), and the accuracy was calculated as the percentage of deviation between the nominal and measured concentration (relative error, RE, %).

The efficiency of the extraction procedure (absolute recovery) was determined by comparing the average concentration obtained from human urine samples submitted to the extraction procedure for 120, 180 and 300 ng mL^{-1} of OXY and 200, 500 and 750 ng mL^{-1} of DEO with samples enriched after extraction for the same concentrations.

A full factorial design was selected to evaluate the robustness of the method. Minitab® 14 statistical software (State College, PA, USA) was used. Three factors were studied at high and low levels: the temperature of the analysis (30 ± 1 °C), the concentration of BGE (50 ± 2 mmol L^{-1}) and the pH of BGE (3.0 ± 0.1). Migration time and area of each peak were the considered effects.

The stability study for OXY and DEO included: influence of freeze (-20 °C) and thaw (22 ± 2 °C) cycles, short-term room temperature (12 h on the bench-top), long-term storage at -20 °C (1 week) and auto-injector (24 h, ambient temperature 25 ± 2 °C). Three replicates (n = 3) of human urine at low (120 and 200 ng mL^{-1} for OXY and DEO, respectively) and at high concentrations (300 and 750 ng mL^{-1} for OXY and DEO, respectively) were prepared to perform the stability test. Concentrations obtained from the stability tests were compared with concentrations obtained from freshly prepared samples (n = 3) at the same concentrations. A one-way ANOVA test was applied with the significance level set at *p*-value ≤ 0.05.

3 Results and discussion

3.1 Selection of the separation parameters

OXY and DEO are basic compounds[11] and the use of a low pH BGE can be an analytical strategy for the analysis of these types of analytes.[37] At this condition, the analytes are positively charged. Furthermore, compounds with pKa > 5 at low pH values are shown completely ionized and do not suffer variations in mobility with small changes in pH.[38] Therefore, the use of low pH (pH < 4) was chosen, which provides negligible electroosmotic flow (EOF)[39] and ensures that all species are protonated.

To investigate the influence of the type of BGE in the separation of OXY and DEO, sodium phosphate buffer, sodium borate buffer, TEA solution and Tris/H_3PO_4 solution were evaluated at an acidic pH. The optimal resolution condition with lowest migration time was achieved with a 50 mmol L^{-1} TEA solution at a pH of 3.0. When the capillary was maintained at 25 °C with an applied voltage of +30 kV, the peaks of OXY and DEO were obtained in 8.18 min and 8.51 min, respectively. In these conditions, the resolution among the peaks of OXY and DEO was 3.3.

Another important parameter to be controlled for the improvement of the separation is the concentration of the BGE. Experiments using the TEA solution with a pH of 3.0 at 15-100 mmol L^{-1} concentrations were carried out to achieve maximum efficiency and resolution. Increasing the ionic strength of BGE can reduce electromigration dispersion; however, higher ionic strength can also lead to an increase in current.[40] As shown in **Figures 1A, B and C**, when the concentration of BGE was increased, a raise was observed in the migration times of OXY and DEO. . This event happens due to an increase in the viscosity of the BGE solutions, which reduces the electrophoretic velocity.[41]

Figure 1D shows the effect of capillary temperature on OXY and DEO resolution. BGE depletion and excessive Joule heating was taken into account during the selection of the final conditions. The increase in the applied voltage to +30 kV resulted an improvement in efficiency and resolution and also contributed to a decrease in the migration times.

Therefore, the electrophoretic conditions established for the analysis of OXY and DEO were a 50 mmol L^{-1} TEA solution at a pH of 3.0 as the BGE with a constant voltage of +30 kV. The temperature of analysis was set at 30 °C and the injection was performed in

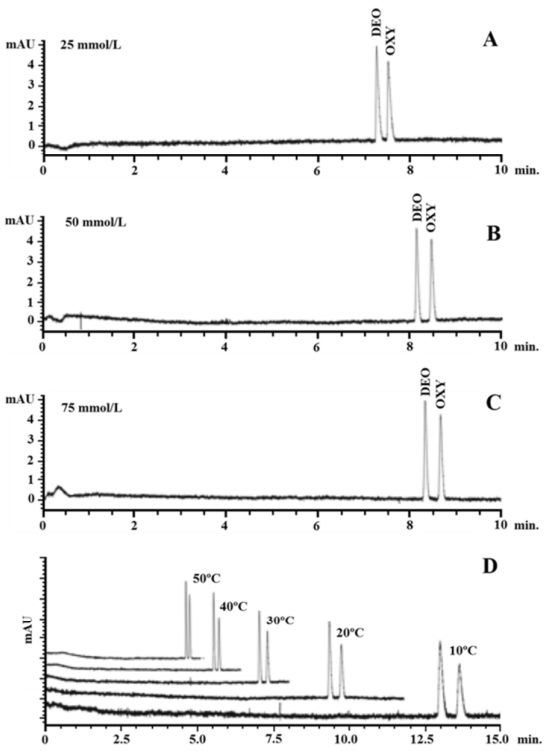

Figure 1. Effect of BGE concentration 25 mmol L^{-1} (A), 50 mmol L^{-1} (B), 75 mmol L^{-1} (C) and temperature (ºC) (D) on migration times. CE conditions: capillary, 50 µm i.d. × 45 cm (effective 36.5 cm); hydrodynamic injection of 50 mbar × 20 s; DAD detection at 204 nm; BGE: 50 mmol L^{-1} of TEA (pH adjusted to 3.0 with H_3PO_4 1.5 mol L^{-1}); applied voltage of +30 kV; temperature of 30°C.

hydrodynamic mode by applying a pressure of 50 mbar for 20 s. Employing the established condition of analysis, reproducibility of the migration times and peak areas were evaluated by five consecutive injections of a unique sample using the same BGE. Under these conditions, RSD values for the migration time and peak area of the analytes were 0.6% and 3.3% for OXY and 0.5% and 1.7% for DEO.

3.2 Optimization of extraction conditions employing DLLME

To optimize DLLME for OXY and DEO extraction from human urine samples, analytical factors that potentially affect the efficiency of this sample preparation were studied, such as selection of the extraction and disperser solvents, volume of the extraction and disperser solvents, extraction time and sample pH.[17, 18, 42-45]

3.2.1 Choice of extraction solvent

The selection of an appropriate organic extraction solvent is of high importance in the DLLME process. It needs to be immiscible in urine, have higher density than that of the sample and have high extraction capability for the analytes.[24, 46, 47]

For this first parameter, the total volume of the extraction/disperser solvents mixture was 500 µL, i.e., 100 µL of solvent extraction and 400 µL of disperser solvent (acetonitrile). The extraction solvents were C_2Cl_4, CCl_4, CH_2Cl_2, $CHCl_3$, C_6H_5Cl and CS_2. Among these, CS_2 was not suitable for being immiscible with acetonitrile and CH_2Cl_2 gave a low volume of the sediment phase, less than 50 µL, likely because of its high solubility in water (13.8 g L^{-1}), and were therefore discarded from the tests.

Regarding the other four solvents evaluated, C_6H_5Cl showed low extraction of OXY and DEO (**Figure 2A**). The CCl_4 was chosen as the extraction solvent because it promoted higher recovery of the compounds and also presented a low RSD. The C_2Cl_4 and $CHCl_3$ solvents were also able to extract OXY and DEO, but they did not show the efficiency of CCl_4 in the interaction and extraction process for both of the analytes.

3.2.2 Choice of disperser solvent

For DLLME, disperser solvents should be miscible with both water and the extraction solvent.[24, 33, 48] In this research, four solvents were evaluated: CH_3CN, CH_3OH, C_2H_5OH and $(CH_3)_2CO$. With the exception of CH_3OH, all of them were similarly efficient at recovering analytes (**Figure 2C**). Thus, based on the value of the area obtained as well as the RSD, CH_3CN was selected.

3.2.3 Influence of volume of the extraction solvent

The extraction solvent volume has great effects on the EF. With an increase of the extraction solvent volume, the final organic phase obtained by centrifugation is increased, resulting in a decrease in the concentration of the target analyte in the organic phase. Although the recovery remains nearly constant, the EF will be decreased.[17] However, small volumes of extraction solvent lead to the formation of small volumes of the sediment phase, which can affect reproducibility. Moreover, the formation of a white solid lipid phase between the aqueous and organic layers, as a result of uric acid and carbamate precipitation at high pH values,[33, 44] makes it difficult to pick-up the extraction solvent from the bottom of the tube[34] and can affect the reproducibility of the extraction method. Therefore, it was chosen to perform tests with larger volumes of solvent (80-140 µL), and results showed that the recovery was slightly greater when 140 µL of carbon tetrachloride was used (**Figure 2B**).

In addition, it was observed that with an increase in the amount of organic solvent came an increase in the volume of the sediment phase obtained (**Figure 2G**). Therefore, it was decided to withdraw a volume of 100 µL from the sediment phase instead of the 50 µL previously selected.

3.2.4 Influence of volume of the disperser solvent

Volumes of 260, 360, 560 and 860 µL of the disperser solvent were evaluated. It was observed that increasing the volume of the disperser solvent did not result in an increase in the recovery of DEO. In addition, the increased volume of the disperser solvent caused a decrease in recovery of OXY (**Figure 2D**), so the acetonitrile volume chosen for DLLME was 260 µL. The lower recovery of OXY can be explained by an increase in the solubility of the analyte in the sample when a larger volume of the disperser solvent is used. This decreases the partition towards droplets of the organic solvent, thus reducing the extraction efficiency.[47]

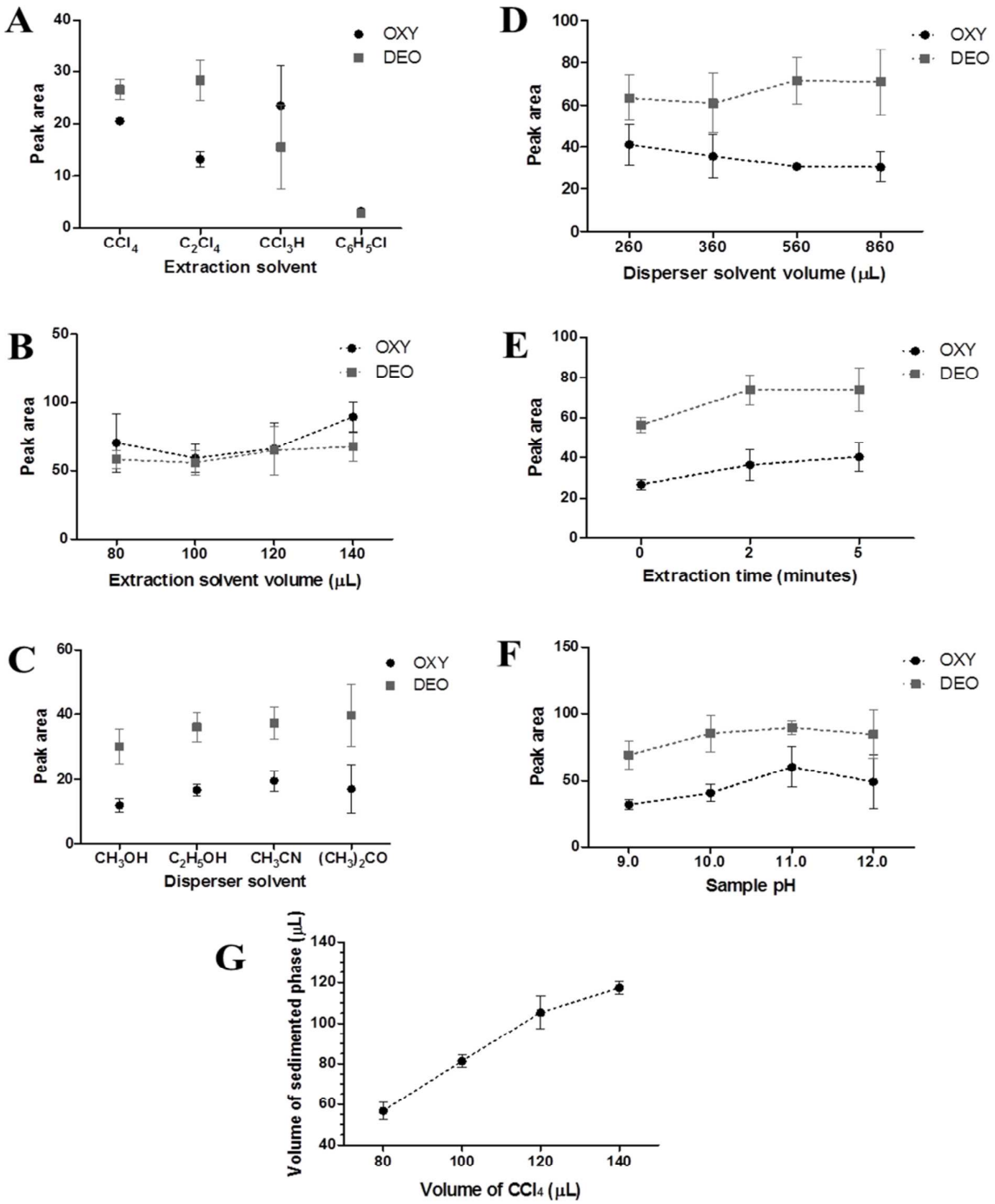

Figure 2. Effect of: (A) extraction solvent type; (B) extraction solvent volume; (C) disperser solvent type; (D) disperser solvent volume; (E) extraction time; (F) sample pH on the DLLME process and (F) effect of the CCl_4 volume at the volume of the sediment phase.

3.2.5 Influence of extraction time

The extraction time was evaluated by leaving the injected solvents in contact with the sample for 0, 2 and 5 min before starting centrifugation. There were no statistically significant differences between the extraction times because the *p* value was above the threshold value of 0.05 ($p = 0.38$), showing that time has no effect on the extraction efficiency (**Figure2E**). The large contact area between the solvent and sample, promoted by the presence of a disperser solvent, allows for a fast mass transfer from the aqueous phase to the extraction phase and an equilibrium state is achieved

without time dependence, which is one of the major advantages of DLLME.[23, 25, 32] Therefore, a 2 min extraction time was maintained during this experiment.

3.2.6 Influence of sample pH

Sample pH is a key parameter of DLLME.[49] It affects both the efficiency and selectivity of the method.[46] In 2009, Melwanki and co-workers reported that the extraction of an analyte from urine samples without converting them to molecular form causes low extraction efficiencies.[47] The analytes are in molecular form when the pH of the aqueous phase is higher than the pKa of the analytes, resulting in a much easier extraction by the extraction solvent than those in ion forms.[33] OXY and DEO are basic compounds[11] with pk_a values of 11.95 ± 0.29 and 8.24 ± 0.25 for OXY and pk_a values of 11.94 ± 0.29 and 7.56 ± 0.10 for DEO. Sample pH values were evaluated at 9, 10, 11 and 12, and the ones that provided the best extraction of the compounds of interest were pH 11 and 12, as shown in **Figure 2F**. There was no significant difference between the amount recovered between them because the *p* value was greater than 0.05 for both OXY (p = 0.51) and DEO (p = 0.67). However, to avoid possible OXY degradation at a pH of 12,[50] whose half-life is 14 min at this pH, a pH of 11 was selected.

3.3 Method validation

The method validation was performed under CE and DLLME optimized conditions and with a urine NaCl addition of 2.5% (w/v). After optimization, the resolution of OXY and DEO were 3.1 with migration times of 7.12 and 7.42 min, respectively. System suitability was assessed by the RSD of the parameters peak area (RSD < 3.5% for OXY and RSD < 2.6% for DEO) and migration time (RSD < 2.1% for OXY and DEO).

Figure 3A shows an electropherogram referring to the human urine blank submitted to DLLME. **Figure 3B** shows an electropherogram obtained from human urine spiked with OXY (120 ng mL^{-1}) and DEO (200 ng mL^{-1}) after the DLLME procedure. As shown, DLLME promotes a good cleaning of the sample matrix because no interference peaks are observed in the migration times of the analytes.

Linear regression analyses were performed by plotting the peak area ratios between drug or metabolite and IS (y) *versus* the theoretical analyte concentrations (x). The method proved to be linear over the concentration range of 90-300 ng mL^{-1} for OXY and 187.5-750 ng mL^{-1} for DEO, with a correlation coefficient of $r > 0.99$. In addition, linearity was evaluated by an ANOVA lack of fit test with a *p*-value higher than 0.29. The LOQs for the validated method were 90 ng mL^{-1} and 187.5 ng mL^{-1} for OXY and DEO, respectively. The RSD (%) and RE (%) were lower than 10% (**Table 1**).

Under optimized conditions, DLLME recoveries were 71.4% for OXY and 60.9% for DEO, with RSD values lower than 10%. In addition, the EF was 30.5 and 26 for OXY and DEO, respectively (**Table 1**). High recoveries are desirable but not mandatory criteria for validation. It is most important to reach a reproducible recovery even when the recovery itself is low.[51] For DLLME methods using biological samples, some recoveries are below 60%[32, 34, 52] and some methods don't show high EF.[44]

The method's precision and accuracy were evaluated by intra- and inter-day assays. The RSDs and REs were lower than 15% (**Table 2**).

Stability tests showed no significant difference for the *p*-values of the freeze and thaw cycles, short-term room temperature, long-term storage at -20 °C and at permanence in the auto-injector **(Table 3**), indicating that analytes are stable under preparation, analysis and storage conditions.

During the determination of method robustness, the effect's significance was evaluated by a Pareto chart that consists of bars with a length proportional to the absolute value of the estimated effect, divided by the pseudo standard error Lenth (Lenth's PSE)[53]. A critical *t*-value for an α of 0.05 was established and the factors' effect, at studied ranges, were not statistically significant (α = 0.05) for most of the responses except for the influence of BGE's pH in the area of OXY.

4 Conclusions

To our knowledge, this paper describes, for the first time, the use of DLLME and CE for simultaneous determination of OXY and DEO in urine. The developed and validated method is very simple, fast and requires negligible organic solvent consumption. DLLME and CE were shown to be efficient techniques to be used for biological samples and offers versatile approaches in analyte enrichment together with simultaneous clean up. Personnel training is necessary to ensure reproducibility. The inconveniences lay on the need of a centrifugation step and that the developed method does not have the required sensitivity to meet the regulatory requirements for the real samples. The somewhat inferior sensitivity of the CE-DAD method can be overcome if fluorescence or MS/MS detection is available.

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Conflict of interest

The authors have declared no conflict of interest.

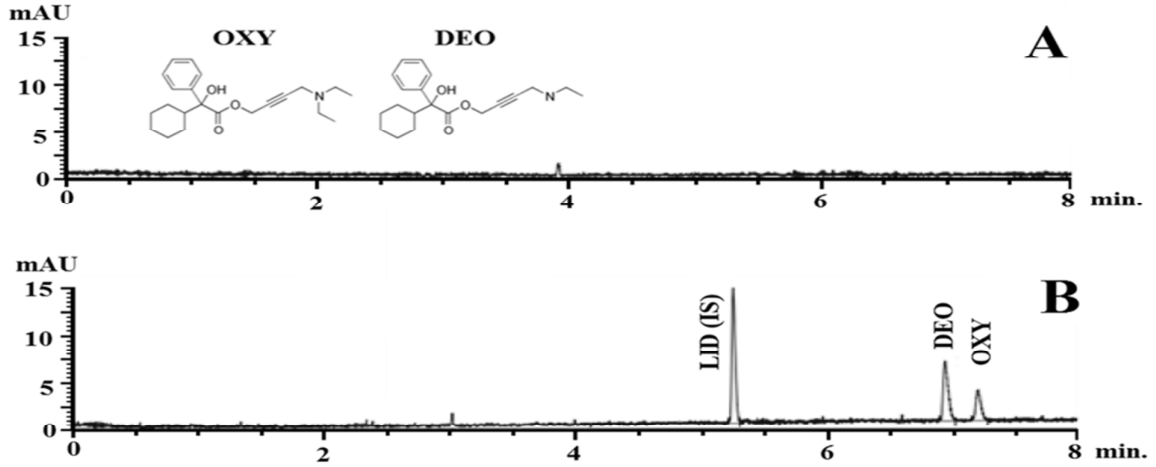

Figure 3. Electropherograms of human blank urine (A) and human spiked urine (B).

Table 1. Linearity, LOQ and recovery of the OXY and DEO method for analysis of urine

Parameters	OXY	DEO
Linear equation[a]	y = 0.0062x – 0.1294	y = 0.0072x – 0.6093
Coefficient of correlation (r)	0.990	0.998
Range (ng mL^{-1})	90-300	187.5-750
Experimental F-value[b]	1.40	0.79
***p* value**	0.29	0.58
LOQ (ng mL^{-1})	90	187.5
Precision (RSD, %)	5.2	2.2
Accuracy (RE, %)	0.9	1.8
Recovery	71.4	60.9
RSD (%)	6.4	8.5
EF	30.5	26.0

[a]Calibration curves were prepared in triplicate (*n* = 3) for concentrations of 90, 120, 150, 180, 240, 270 and 300 ng mL^{-1} for OXY and 187.5, 200, 250, 375, 500, 600 and 750 ng mL^{-1} for DEO; y = Ax + B, where y is the ratio between the analyte's peak area and the IS's peak area, A is the slope, B is the intercept, and x is the concentration of the measured solution in ng mL^{-1}; [b]Experimental F-value < $F_{crit, 95\%}$ = 4.28.

Table 2. Precision and accuracy of the method for OXY and DEO analysis

Parameters	OXY			DEO		
Nominal concentration (ng mL^{-1})	**120.0**	**180.0**	**300.0**	**200.0**	**500.0**	**750.0**
Intra-day (n[a] = 5)						
Analyzed concentration (ng mL^{-1})	113.7	182.8	278.9	193.6	507.4	745.0
Precision (RSD, %)	7.7	12.2	10.4	7.9	10.1	5.8
Accuracy (RE, %)	-5.2	1.6	-7.0	-3.2	1.5	0.7
Inter-day (n[b] = 3)						
Analyzed concentration (ng mL^{-1})	113.9	181.9	295.4	196.4	505.1	737.0
Precision (RSD, %)	6.7	11.2	10.1	6.7	7.3	6.0
Accuracy (RE, %)	-5.1	1.1	-1.5	-1.8	3.0	-1.7

[a] n = number of determinations; [b] n = number of days.

Table 3. Stability test for the developed method

		OXY		DEO	
Nominal concentration (ng mL^{-1})		**120**	**300**	**200**	**750**
Freeze-thaw cycle stability (n = 3)[a]	*p* value[b]	0.32	0.76	0.16	0.78
	RSD (%)	14.1	3.4	3.4	3.2
Short-term bench-top stability (n = 3)[a]	*p* value[b]	0.31	0.13	0.66	0.60
	RSD (%)	7.0	11.7	10.6	3.3
Long-term stability (n = 3)[a]	*p* value[b]	0.06	0.83	0.59	0.78
	RSD (%)	12.1	12.5	9.6	13.5
Auto-injector (n=5)[a,c]	*p* value[b]	0.61	0.91	0.70	0.97
	RSD (%)	13.5	4.4	11.0	7.7

[a]n = number of determinations for samples submitted to stability test;
[b]Level of significance set at $p < 0.05$;
[c]24 h, 25 ± 2 ºC.

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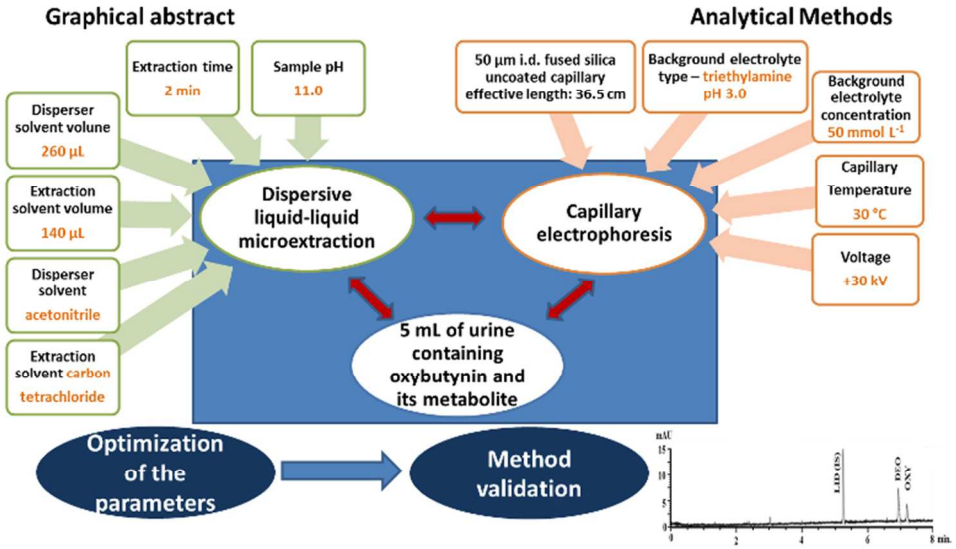

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