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Article

Xanthine Derivatives Quantification in Serum by Capillary Zone Electrophoresis

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A capillary electrophoresis method was developed to quantify caffeine and theophylline, xanthine derivatives with bronchodilator activity. Buffer concentration, pH and applied voltage were optimized using a central composite design-face centred. Separation conditions were: silica capillary tube, 75 μm (i.d.) and 61 cm (total length); absorbance detection, 280 nm; borate buffer, 20 mM, pH 9.0; applied voltage, 25 kV and 1 psi injection/8 s. Validation was performed in blank serum following the International Conference Harmonization guidelines: resolution (peaks without overlapping), linear range (0.125–50 μg/mL; $r^2 > 0.9999$), limits of detection and quantification (10; 20 and 33; 66 ppb for caffeine and theophylline, respectively), intra- and inter-day precision (Relative standard deviation lower than 1.9%) and accuracy (98–101%). Migration times were <8 min. This method is simple, specific and suitable and reaches high label claims (98.7–100.4%) in pharmaceutical formulations analysis. Moreover, the method was applied to the monitoring of the analytes in serum of patients.

Introduction

Caffeine (MW = 194.19 g/mol; log *P*o/w = 7) and theophylline (MW = 180.164 g/mol; log *P*o/w = −2) are purine bases whose structures are similar to dimethyl xanthines (Figure 1). Both compounds are weak base and acid (1), with pKa(caffeine-H^+)=0.6: pKa (caffeine) = 14; pKa (theophylline-H^+) = 3.5 and pKa (theophylline) = 8.7. These xanthine derivatives are used as bronchodilators. However, patients treated with identical dosages of these compounds present large differences in plasma drug concentrations, which may imply a different therapeutic effect. Such variability requires individualized treatment monitoring (therapeutic drug monitoring). Theophylline is a powerful smooth muscle relaxant that is frequently used for the treatment of asthma and chronic obstructive lung disease in acute and chronic settings and apnea in premature infants. It also encourages diaphragmatic contractility and mucociliary clearance, assists cardiac functions and decreases pulmonary artery pressure (2, 3). However, theophylline also shows several significant adverse effects. Appropriate dosing is essential as theophylline has a narrow therapeutic index which falls in the range of 5.4–18 mg/L (2, 3). There are other methylxanthines, such as caffeine, but they are not as potent as theophylline. In short, theophylline and caffeine belong to the group of purines that can cause various physiological effects, such as stimulation of the central nervous system, gastric acid secretion and diuresis. They have also been involved in various disorders, including heart disease, carcinogenesis, kidney malfunction and asthma (2). Therefore, the monitoring of these compounds in serum is useful to relate methylxanthine concentrations with effects (positive or toxic) in human health and establish their pharmacokinetics (3).

Several capillary electrophoresis (CE)-based methodologies have been used to analyze caffeine and/or theophylline in different real samples (4–13). Some papers describe the separation of caffeine or theophylline in tea (8, 10–14), pharmaceuticals (5, 11–13) and biological samples (6, 7, 15). However, there are a very few research articles about the simultaneous determination of caffeine and theophylline in a single run because they show a similar behavior and are difficult to separate by CE (6, 8).

CE methods offer several advantages over HPLC methods. CE is a highly efficient separation system requiring low quantity of organic modifier for separation and a low amount of sample. Usually, CE sample preparation and analysis times are faster than those in HPLC. CE methods can generate comparable accuracy and precision to HPLC for pharmaceutical purposes (16). Despite these advantages, CE is still used less in clinical and industrial routine analyses, because these laboratories prefer to use liquid chromatography instrumentation.

Experimental designs are based on the variation of several variables at the same time rather on varying each one separately while keeping the others constant. This multivariate approach leads to a response model in which the relationship of each variable (i.e., factor) toward the response along with the interactions between the factors is shown. Replicated center points can be added to provide protection against curvature (quadratic effects) and to obtain an independent estimate of error (17–19).

The aim of the present work was to develop, optimize and validate a rapid CE method that allows the determination of caffeine and theophylline together, individually or mixed with other drugs in pharmaceutical formulations. The validation must be performed following the requirements of International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) indications in terms of selectivity, specificity, linearity, sensitivity, the detection and quantification limits (LOD, LOQ), precision, accuracy and robustness (20).

Experimental

Reagents and chemicals

Caffeine and theophylline were purchased from Sigma (St Louis, MO, USA). The reagents used to prepare the background

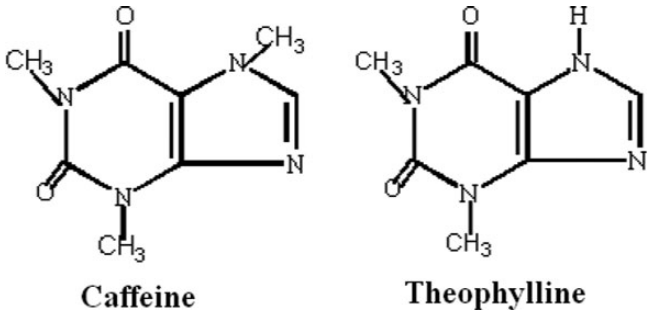

Figure 1. Structures of caffeine and theophylline.

electrolyte (BGE) were of analytical grade: boric acid, sodium acetate, acetic acid, sodium hydroxide (99% purity, Merck, Darmstadt, Germany) and *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES) (Sigma). The flexible fused silica capillary was obtained from Polymicro Technologies (Phoenix, USA). The standard solutions and the BGE were filtered through 0.22 μm nylon membranes of 12 and 45 mm i.d. (Micron Separations, Westboro, MA, USA), respectively. Distilled-deionized water (Barnstead, Sybron, Boston, MA, USA) was used throughout. The pharmaceutical formulations were provided by the "Hospital General" (Castelló, Spain).

Instrumentation and CE conditions

CE runs were performed with a Beckman P/ACE System MDQ (Beckman Instruments, Inc., Fullerton, CA, USA) equipped with a DAD detector. A PC computer connected to the instrument through the Beckman 32 karat software was used for instrumental control and chromatographic data acquisition. pH measurements were performed with a GLP 22 (Crison, Barcelona, Spain), provided with a combined Ag/AgCl/glass electrode. The vortex shaker and ultrasonification unit were purchased from Selecta (Barcelona). The SPSS program, version 18 (SPSS Inc. Chicago, IL), was used for data processing.

The method shows high resolution and allows separation of both xanthine derivatives in 8 min under optimized conditions: a silica capillary, 75 μm (i.d.) and 61 cm (total length, 50 cm, effective length), a diode array detector (DAD) at 280 nm, 20 mM borate buffer (without organic solvent) at pH 9, 25 kV separation voltage, 20 μA amperage, 25°C temperature, and 1 psi/8 s hydrodynamic injection.

Capillary conditioning

The polyimide coating of the capillary was partially removed by burning at the point of detection, and the uncovered portion of the capillary was aligned on the detector block. The capillary was conditioned daily with an initial wash cycle consisting of 1 M NaOH for 15 min, deionized water for 10 min and running an electrolyte for 5 min. Between the two injections, the capillary was washed with 0.1 M NaOH for 2 min, deionized water for 1 min, and the electrolyte was run for 3 min at 20 psi. The separation buffer was refreshed after hundred runs. On a daily basis, and before and after finishing the experiments, the capillary was washed with 1 M NaOH for 10 min, deionized water for 10 min and was air purged for 3 min.

Sample preparation

A standard stock solution of caffeine and theophylline, containing 500 μg/mL of each compound, were prepared by dissolving the analytes in 5% of methanol and then filling up the flask with ultrapure water. Stock solutions were stored in amber flasks at 4°C.

Pharmaceutical dosage forms were analyzed for caffeine and theophylline according to the method. In all cases, the dosage forms were capsules, tablets or drageés. For assay preparation purposes, the contents of 10 tablets or capsules were powdered using a pestle in a mortar, and the powder equivalent to one average tablet weight was taken. A weighted portion of the powder equivalent to the label claim of formulation was transferred to a 25 mL volumetric flask. The powder was fully dissolved in 5% methanol and was then diluted with water up to the mark. The solutions were stored in colored flasks at 4°C.

Samples and pharmaceuticals formulation were analyzed as follows: 0.5 mL of serum or solved pharmaceutical formulations (performed as indicated in the previous paragraph) was added to a volumetric flask of 5 mL, then filled up with buffer and filtered. In the case of spiked samples, the appropriate amount of standard (depending on the spiking concentration) was added before filling up the flask. The solution in the micellar media and the filtration assures that no particles are introduced in the electrophoretic system.

Serum samples, corresponding to volunteer patients ($n = 20$) treated with the xanthine derivatives and healthy subjects (blank), were provided by the Servei d'Extraccions Sanguinies (a blood extraction survey) of the Hospital General (Castelló, Spain) with permission of the Ethics Commission. In both cases, serum samples were diluted by the appropriate amount of buffer and injected.

Results and discussion

Optimization of the experimental conditions

Electrophoresis separation depends on many experimental parameters (section capillary conditioning). Among them, buffer concentration, pH and applied voltage strongly influenced the resolution, thus were studied to optimize the separation of caffeine and theophylline (17). The minimum and maximum values for these parameters were: buffer concentration (10–50 mM), pH (3–11) and applied voltage (10–30 kV).

An interpretative strategy was chosen to carry out the optimization procedure: a central composite face design (CCF) based on varying all the factors simultaneously at a limited number of factor levels. The CCF consists in a combination of factorial design and additional design (star design) and, in this case, the star points are at the center of each face of the factorial space. It offers high precision with a minimum number of injections (only 15 runs are necessary), and it enables factor interactions to be detected. The selection criteria of the separation conditions were the achievement of the maximum separation of the analytes in the mixture within a minimum time, thus resolution and migration time, were parameters of primary importance to assess the suitability of the experimental result (17). Surface response of the resolution versus pH and buffer concentration of buffer running at a constant voltage is shown in Figure 2.

The optimized electrophoretic conditions were pH 9.0, molarity 20 mM and voltage 25 kV. Under these conditions, migration time for caffeine and theophylline were: 4.3 ± 0.1 and 7.5 ± 0.2 min.

Figure 3A shows the electropherogram obtained under the optimum conditions for caffeine and theophylline determination. However, Figure 3B shows an injection of acetone which was used as an electroosmotic flow (EOF) marker: under the optimum conditions, the EOF did not interfere with the determination of the two analytes.

Method validation

Method validation establishes that the method performance characteristics are suitable for the intended use. The entire validation was performed using blank serum. Validation was performed by studying the specificity, selectivity, linearity, accuracy, precision, sensitivity, limit of detection (LOD) and quantification (LOQ) and robustness, according to ICH guideline (20). The method was also validated by comparing it with other work previously reported (7). The electropherogram obtained by the analysis of serum sample free of xanthines is shown in Figure 4A.

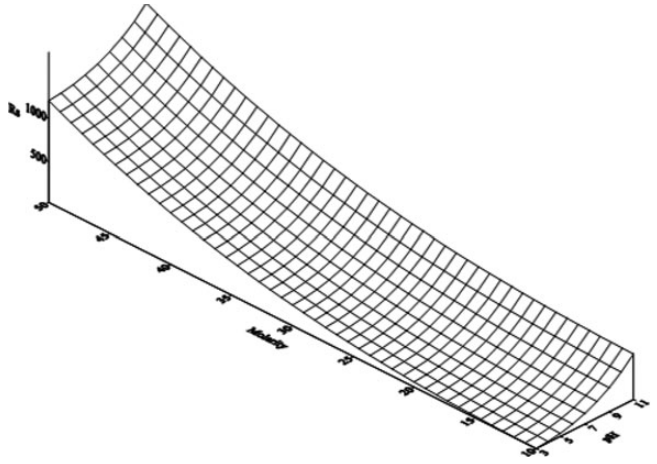

Figure 2. Surface response of the resolution: (A) versus pH and buffer concentration of buffer running at a constant voltage.

Calibration curves were constructed by spiking blank serum with varying amounts of the analytes from the standard stock solutions to give a calibration range from 0.125 to 50 μg/mL. Curve parameters were obtained by plotting the peak areas *vs.* the concentrations by least-square linear regression. To study the variability of the calibration parameters, curves were obtained for 5 days over a 2-month period for a different set of standards. The calibration curves were as follows:

$$A = (4220 \pm 15)[\text{caffeine}] + (12 \pm 2)$$
$$A = (940 \pm 4)[\text{theophylline}] + (2 \pm 0.3).$$

In both cases $r^2 > 0.9999$ and the concentrations [X] are expressed as μg/mL. These calibration curves were reliable than previously reported (7), which r^2 was 0.9987.

The detection limits (LOD) were taken as three times the noise level, in this case 10 and 20 ng/mL for caffeine and theophylline, respectively. The electropherogram obtained by the analysis of blank serum spiked at LOD levels of each xanthine is shown in Figure 4B. The quantification limit (LOQ), taken as 10 times the noise level, were 33 and 66 ng/mL for caffeine and theophylline, respectively (21). The sensitivity was found higher than previously reported (7), in which LOD was 1 μg/mL.

The intra-day precision and accuracy were determined by analyzing spiked serum at three different drug concentrations (10 replicates) at the same day: 2; 10 and 20 μg/mL. The spiking concentration has been chosen as the normally found in real samples, not depending on the interval range of linearity. The precision was the standard deviation of the obtained areas of electropherogram peaks. The accuracy was the ratio between the analyte amount calculated by the method and the spiked amount. Inter-day precision and accuracy were taken by analyzing spiked samples by the same way as intra-day studies, but over 5 consecutive days. Results are shown in Table I. The results, expressed as relative standard deviations (RSD, %), and accuracy, were in the 0.25–1.83% and 98–101% ranges, respectively, indicating excellent precision, accuracy and assessing the present method's ability to be used in the monitoring of the xanthine

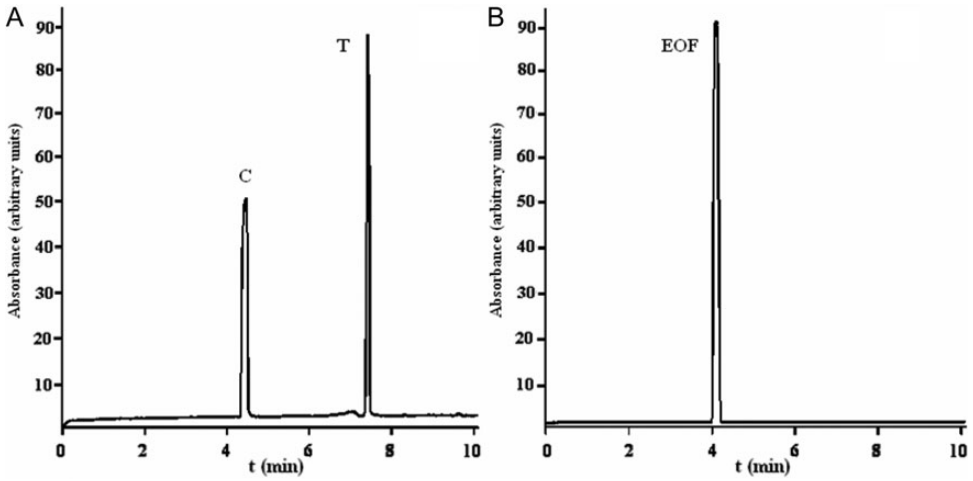

Figure 3. Electropherograms showing (A) the separation of caffeine (C) and theophylline (T) (both 15 μg/mL) and (B) EOF marker, using 20 mM borate pH 9, 25 kV separation voltage and UV detection λ = 280 nm.

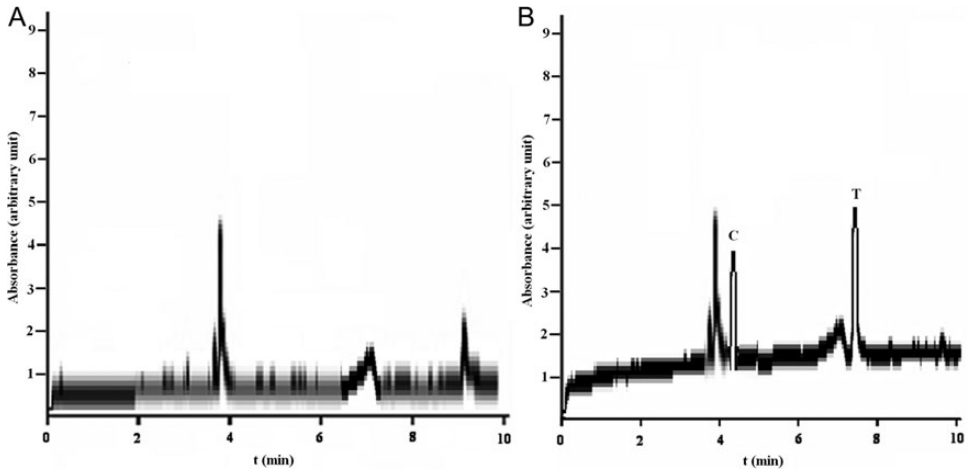

Figure 4. Electropherograms obtaining by the analysis of serum: (A) blank and (B) spiked at 10 ng/mL of caffeine and 20 ng/mL theophylline. Analysis conditions as in Figure 3.

Table I.
Intra-day and Inter-day Precision (RSD %, $n = 10$) and Accuracy (%, $n = 10$) at Three Different Concentrations (μg/mL): $c_1 = 2$, $c_2 = 10$, $c_3 = 20$

Compound	Intra-day precision			Inter-day precision[a]		
	c1	c2	c3	c1	c2	c3
Caffeine	0.25	0.5	1.00	0.86	0.77	1.83
Theophylline	0.38	0.93	0.88	0.98	1.74	1.08
	Intra-day accuracy			Inter-day accuracy[a]		
	c1	c2	c3	c1	c2	c3
Caffeine	98.2	99.1	100.5	98.6	99.3	99.8
Theophylline	100.9	98.8	99.5	98.4	100.2	100.3
Migration time (min)	Intra-day (RSD, %)			Inter-day (RSD, %)		
Caffeine	4.31 ± 0.05 (0.92)			4.35 ± 0.07 (1.6)		
Theophylline	7.54 ± 0.06 (0.80)			7.48 ± 0.09 (1.2)		

[a] $n = 5$.

Table II.
Determination of Caffeine and Theophylline in Pharmaceutical Preparations

Commercial name and manufacturer	Composition	Label claim (%) ($n = 5$)
Analgilasa, Lasa Laboratorios, Spain	Caffeine 30 mg, paracetamol 500 mg, codeine phosphate 10 mg, excipient sufficient amount (c.s.)	99.9 ± 0.3
Coricidin, Schering-Plough, SA, Spain	Caffeine 30 mg, chlorphenamine maleate 4 mg, salycilamide 190 mg, Vitamin C 50 mg.	100.4 ± 0.6
Desenfriol, Fardi, SA, Spain	Caffeine 32.4, chlorphenamine maleate 2 mg, phenylephrine HCl 12.2 mg, acetylsalysilic acid 388.8 mg, excipients: saccharose 121 mg and others c.s.	100.2 ± 0.5
Rinomicine comprimidos, Fardi, SA, Spain	Caffeine 30 mg, chlorphenamine maleate 4 mg, paracetamol 150 mg, salycilamide 150 mg, phenylephrine HCl 10 mg, excipient c.s.	100.1 ± 0.8
Asmo-Hubber retard, ICN Ibérica SA, Spain	Theophylline 300 mg, excipients c.s.	98.7 ± 0.2
Theo - Dur 200, Pharmacia Farmitalia, Spain	Theophylline 200 mg, excipients: lactose 62.5 mg and other c.s.	99.2 ± 0.5

Table III.
Determination (concentration ± standard deviation in μg/mL) of the Xanthine Derivatives in Serum of Patients

Patient	Caffeine	Patient	Theophylline
C-1	15.28 ± 0.02	T-11	11.24 ± 0.08
C-2	30.65 ± 0.03	T-12	15.63 ± 0.05
C-3	12.32 ± 0.04	T-13	8.95 ± 0.09
C-4	2.14 ± 0.05	T-14	14.8 ± 0.2
C-5	31.63 ± 0.07	T-15	13.4 ± 0.3
C-6	31.24 ± 0.05	T-16	7.25 ± 0.06
C-7	11.81 ± 0.06	T-17	24.75 ± 0.06
C-8	33.32 ± 0.09	T-18	12.57 ± 0.07
C-9	22.54 ± 0.03	T-19	9.15 ± 0.09
C-10	32.27 ± 0.04	T-20	4.87 ± 0.08

derivatives. The reproducibility of this methodology was higher than previously reported (7), in which precision was <2%. Intra- and inter-day precision for migration time of analytes were also calculated, obtaining a precision of <2.7%.

Specificity is the method's ability to accurately identify the analyte in the presence of all the potential sample components. The selectivity criterion for an assay method is that the analyte peaks will have an electrophoretic baseline with a suitable resolution with all the other sample components (analytes and non-analytes). To study the matrix effects of the possible co-eluting compounds and the EOF on the pharmaceuticals, 10 blank samples were analyzed by adding acetone as the EOF marker (Figure 3B). The EOF appears before the two analytes, and there were no peaks near the migration time of either caffeine or theophylline. Even if the caffeine is neutral under these conditions, it shows a slight delay from the EOF, due to the interaction between the amine groups of the analyte with the silanol groups of the capillary (22). Yet under the optimized operating conditions, the peaks resolved (Figure 3A) and none of the other components present in the pharmaceuticals preparations (as described in Table II) overlapped the peaks corresponding to caffeine or theophylline. Obviously, the results obtained for patients can be altered by the consumption of other sources of xanthine derivatives, as coffee and chocolate.

The robustness of the method was evaluated by measuring the migration time and peak area for slight variation of the following electrophoresis conditions: borate buffer (19–21 mM), wavelength (275–285 nm), voltage (24–26 kV) and capillary length (49–51 cm effective length). The measures were performed by injecting three replicates of a standard solution of 10 μg/mL of

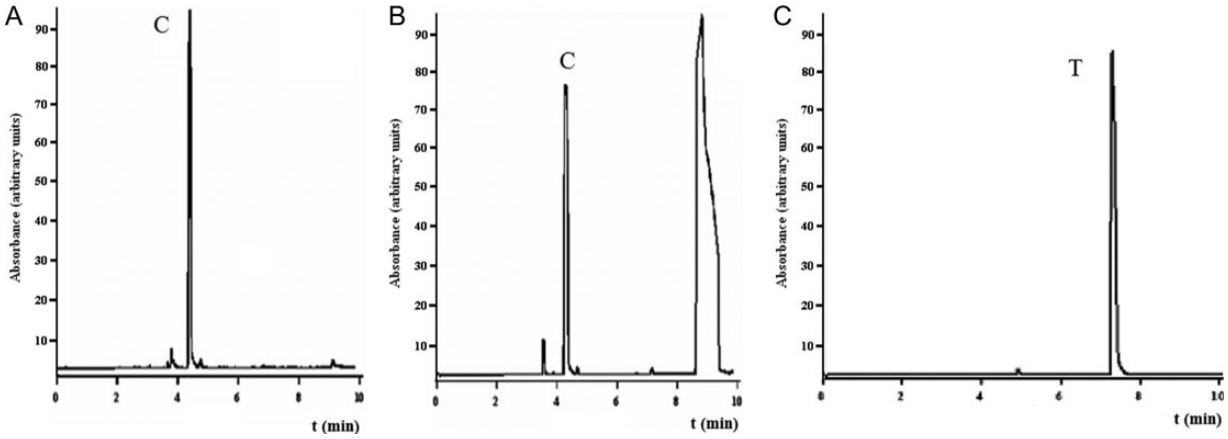

Figure 5. Electropherograms recorded for caffeine (C) (A and B) and theophylline (T) (C) in serum samples of patient. Analysis conditions as in Figure 3.

caffeine and theophylline in serum samples. The RSDs (%) obtained for changes in the migration time, peak area and resolution, corresponding to the influence of these parameters were calculated, being <5.2%. As conclusion, the variations in all the studied parameters had no significant effect on migration time, peak area or resolution and method is robust using the recommended conditions: 20 mM borate buffer at pH 9, UV detection at 280 nm, voltage 25 kV and effective capillary length of 50 cm.

Known amounts of xanthine derivatives, within the calibration range: 5; 25 and 50 μg/mL, were spiked in serum samples and results obtained using the method here described were compared with results obtained with the method previously reported. Results given for both methods are correlated, being the slopes, intercepts and correlation factors of: 0.97, 0.02 and 0.99 for caffeine and 0.99, 0.06 and 0.98 for theophylline. These results indicate that the optimized and validated method is reliable for the determination of the xanthine derivatives in biological samples. Moreover, the comparison of the methodologies indicates that the present work provides better results.

This methodology is fast, easy and provides high sensitivity, precision, accuracy, repeatability and robustness. It can also be used as routine analysis to drug monitoring.

Determination of xanthine derivatives in pharmaceutical formulations and serum samples

The CE method was applied to the determination of caffeine and theophylline in pharmaceuticals, spiked serum samples and monitoring of the xanthine derivatives in serum samples. The analysis was performed 24 h after the ingestion of the pharmaceutical formulation.

Three different pharmaceutical formulations for caffeine and two for theophylline were analyzed. The excipients are different for each pharmaceutical formulation and do not interfere with the analytes. The results are shown in Table II. Label claims were in the 98.7–100.4% range, thus showing the suitability of the methodology for the determination of the xanthine derivatives in pharmaceutical samples.

The method was applied to the determination of xanthine derivatives to patients, being treated with Desenfriol (containing caffeine), and Theo-Dur 200 (containing theophylline). The results are shown in Table III. Figure 5A and B shows the electropherograms obtained while determining caffeine (C) and Figure 5C contains theophylline (T) under the optimum conditions described herein. A poorly defined peak appears at 9 min, without interfering with the analytes, and no metabolites of caffeine and theophylline were detected. The methodology has been found able to analyze serum of patients taking pharmaceutical formulation containing caffeine or theophylline. It can be also useful to determine if the patients are taking food containing one or both of the studied analytes, which can interfere with the medical treatment.

Conclusions

The CE procedure developed herein, based on an experimental design, is straightforward, sensitive, simple and fast for the determination of caffeine and theophylline in pharmaceutical samples and serum samples. The experimental conditions were: 20 mM borate buffer at pH 9, UV detection at 280 nm, voltage 25 kV and effective capillary length of 50 cm. Under these conditions, analytes separated in <8 min. The methodology has been successfully validated according to ICH indications in terms of specificity, selectivity, linearity, LOD, LOQ, accuracy, precision and robustness. Thus, the method could prove suitable for quality control analyses of caffeine and theophylline in pharmaceuticals and for its monitoring in serum samples.

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