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Determination of sildenafil citrate and its main metabolite by sample stacking with polarity switching using micellar electrokinetic chromatography☆

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Abstract

Micellar electrokinetic capillary chromatography (MEKC) coupled with sample stacking and polarity switching was investigated for the determination of Viagra (sildenafil citrate, SC) and its metabolite (UK-103,320, UK) in human serum in the concentration range of clinical interest. Human serum samples spiked with SC and UK were eluted with methanol from a C_{18} cartridge, the extract was evaporated and regenerated in a solution that contained 1 m*M* phosphate buffer (pH 12.3) and 20% methanol. The MEKC separation was performed using an injection time of 275 s, a polarity switching time of 93 s, a phosphate buffer, (pH 12.3, 15 m*M*) containing 25 m*M* sodium dodecyl sulfate as separation electrolyte and a fused-silica capillary. The analysis takes about 6 min and gives satisfactory inter-day precision with respect to migration times and linear responses over the 80–900 ng/ml concentration range investigated for SC and UK. Intra-day RSDs ($n=4$ graphs) for the slopes of the calibration graphs were 4.86% for SC and 3.50% for UK. Inter-day RSDs for the slopes were 4.37% for SC and 5.39% for UK. Detection limits ($S/N=3$) were about 17 ng/ml for both compounds in human serum. A 1-ml volume of blood serum was necessary to do this determination.

Keywords: Sample stacking; Sildenafil citrate; Viagra; UK-103,320

1. Introduction

It is well know that capillary electrophoresis (CE) can efficiently and rapidly separate anionic, cationic and even neutral species. There is growing interest and research regarding its applicability in diverse areas, especially in biological and biochemical fields.

Generally, spectrophotometric detectors such as UV detectors are applied in CE whereby the UV signal is expected to follow the Beer–Lambert law. Because the optical pathlength is very short (typical internal diameters of the capillaries are around 50–75 μm), the injected sample volumes are low (typical volumes are a few nanoliters) and the UV–Vis absorptivity of most of the products analysed is poor, the detection limits are high compared with high-performance liquid chromatography (HPLC) where much longer optical path lengths are generally applied.

Several approaches can be adopted to improve the

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sensitivity of CE in biomedical applications, in particular for the quantification of drugs at the low concentration. The two main strategies are: (a) increasing the amount of analyte present in the capillary, and/or (b) improving the sensitivity of the detector. The first strategy involves using either chromatographic means, such as offline concentration, on-capillary membrane preconcentration devices, or electrophoretic techniques such as sample stacking [1–4] and field-amplified sample injection (FASI) [5–9]. Detection improvements in the second strategy include cell design (bubble and Z-type) [10–12], and the use of alternative detection systems such as laser-induced fluorescence and mass spectrometry (MS). When alternative detectors are unavailable, FASI appears to be the preferred method for achieving high sensitivity.

Due to in-depth studies in the early 1990s by Burgi and Chien [2,3,13], on-line sample concentration by sample stacking is now a well known way to improve UV detection sensitivity in electrophoresis. Sample stacking occurs at the interface between the low conductivity sample and high conductivity separation zones. Sample ions experience a low electric field when they enter the separation zone, which will cause an abrupt drop in their electrophoretic velocities and create a thin, focused zone of the injected samples. In sample stacking with hydrodynamic injection, long injection of sample solutions prepared in a low conductivity medium is better to maximize the sensitivity enhancements. In neutral to basic background electrolyte solutions, a polarity-switching step is a must to remove or minimize the dispersive effect caused by the mismatch of local electroosmotic flow (EOF) velocities. When the reverse polarity step is applied, the sample buffer is completely removed from the column. After that, the polarity of the electrodes is switched again.

Reviews have been published in the last few years about the preconcentration methods used in CE. Osbourn et al. [14] have recently presented a review of on-line preconcentration methods that are employed in CE and micellar electrokinetic capillary (MEKC). This paper discusses the basic concepts of each technique and the practical applications, advantages and limitations.

Beckers and Bocek [15] have also presented a review about sample stacking in capillary zone electrophoresis (CZE) with emphasis on basic laws regulating the electrophoretic processes and the properties and migration behaviour of moving boundaries in different electrophoretic modes.

The two main injection methods, hydrodynamic and electrokinetic, were compared for sample zone length, flow profile, reproducibility and matrix effects in a recent review [16].

Different strategies to improve the sensitivity in CE for the analysis of drugs in biological fluids at very low concentration levels were compared in Hempel's review [17].

Viagra, used in oral therapy for erectile dysfunction, is the citrate salt of sildenafil [CA 139755-83-2], a selective inhibitor of cyclic guanosine monophosphate (cGMP) specific phosphodiesterase type 5 (PDE5).

Sildenafil citrate (SC) is designated chemically as 1-{[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1-H-pyrazolo[4,3-d]pyrimidin-5-yl)-4-ethoxyphenyl]sulfonyl}-4-methylpiperazine citrate and has the structural formula shown in Fig. 1.

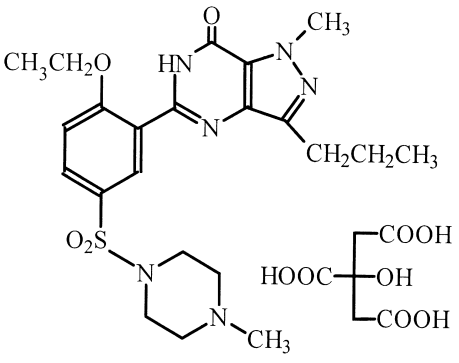
UK-103,320 [CA 139755-82-1] (UK) appears to have a similar potency to sildenafil and hence may contribute to pharmacological effects. Quantification of both compounds is therefore essential during the evaluation of the parent drug (Fig. 1). Both sildenafil and UK-103,320 have basic functional groups with a pK_a value of 8.7, although a weak acidic moiety is also present on the parent compound. Difficulties may arise during the analysis of compounds with basic properties.

An isocratic HPLC method [18] comprising acetonitrile–phosphate buffer–water (28:4:68, v/v) with detection at 230 nm was utilised for routine analysis of SC in pharmaceutical products.

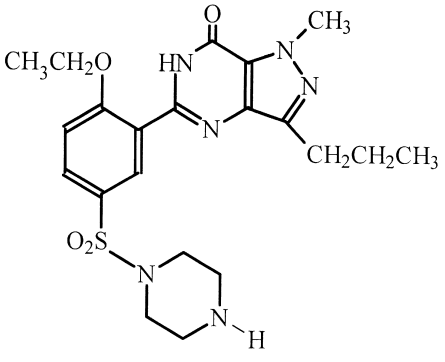
An assay for the simultaneous determination of sildenafil and its metabolite (UK-103,320) using automated sequential trace enrichment of dialysates (ASTED) and HPLC has been also proposed in the paper.

Adsorptive stripping voltammetry has been demonstrated to be a useful technique for the study and determination of many molecules of biological importance [19]. This technique was proposed for the determination of SC in pharmaceutical products [20].

In a previous paper we have proposed MEKC for the determination of Viagra and its metabolite in



Sildenafil Citrate



UK-103,320

Fig. 1. Structures of sildenafil citrate and UK-103,320.

human serum [21] in a concentration range for both compounds of 0.90–4.60 mg/l.

In this paper, we propose a rapid and sensitive method using sample stacking with polarity switching in combination with MEKC for the determination of SC and its metabolite.

2. Experimental

2.1. Materials

All solvents and reagents were of analytical-reagent grade unless otherwise indicated. Sildenafil citrate and its metabolite UK 103,320 were obtained from Pfizer (Sandwich, Kent, UK). Standard solutions (100.0 mg/l) were prepared in methanol and stored at 4 °C. The serum extracted solutions of SC and UK were prepared daily in a solution that contained 1 m*M* phosphate buffer and 20% of methanol.

A 15 m*M* phosphate buffer (pH 12.3) and 25 m*M* sodium dodecyl sulfate (SDS) was used as separation electrolyte and was prepared daily. The set of separation vials was changed after six runs.

2.2. Serum samples for method evaluation

Fresh human serum was obtained from three authors of this paper. Aliquots of human serum were stored at −18 °C and defrosted immediately prior to use. Linearity and intra-day and inter-day precision were evaluated by analyzing aliquots from two different lots of human serum, spiked with SC and UK.

One linearity experiment was carried out on days 1, 2, 3 and 4 of the study. On day 1, four calibration curves were obtained. Linearity and intra-day precision were assessed from the four linearity experiments carried out on the first day. Linearity and inter-day precision were evaluated using the calibration lines from days 2, 3 and 4, in addition to the first calibration line obtained on day 1.

2.3. Human serum pretreatment

The extraction of SC and UK from human serum was performed on a reversed-phase cartridge C_{18} (Sep-Pak Plus, Waters, Milford, MA, USA). The cartridge was conditioned before use by means of 5 ml of methanol, followed by 5 ml of 10 m*M* potassium phosphate monobasic–sodium phosphate dibasic buffer solution, pH 7.0.

Human serum (0.5–2.0 ml) was then slowly loaded onto the conditioned cartridge. After wards, the cartridge was washed with 8 ml of pH 7.0 buffer solution. Then SC and UK were eluted with 1.5 ml of methanol. The volume of human serum was varied to increase sensitivity in the drug determination.

After total evaporation of methanol, the samples

were regenerated with 500 μl of 1 m*M* of phosphate buffer (pH 12.3) which contained 20% methanol. Very low ionic strength in the serum samples with respect to the ionic strength to the separation buffer is necessary to obtain effective stacking (the concentration ratio between sample and separation electrolyte should be 1/10 or less).

2.4. Apparatus and operating conditions

A Beckman P/ACE System MDQ (Fullerton, CA, USA) CE system equipped with a diode-array detector was used. Beckman CE Software controlled the system. Separation was carried out on a 60 cm (50 cm to the detector)×75 μm I.D. fused-silica capillary housed in a cartridge with a 800×100 μm detector window. The wavelength selected for the electropherograms was 230 nm.

The capillary was conditioned prior to its first use by rinsing with 0.5 *M* NaOH for 20 min, water for 10 min and lastly phosphate buffer (pH 12.3, 15 m*M*) with 25 m*M* of SDS for 10 min. At the start of each sequence, the capillary was washed with the separation buffer. All separations were conducted using 2-ml vials for rinsing and washing and 200-μl polypropylene vials as sample vials. All vials were refrigerated at 10 °C inside the equipment and the capillary electrophoresis temperature was 25 °C. The rinse step was carried out using vials different from the separation vials in order to keep the level of buffer constant in the anodic separation vial.

2.5. Procedure

The capillary was filled with the separation buffer for 2 min. A large-volume sample was injected for 275 s at 0.5 p.s.i. (1 p.s.i.=6894.76 Pa). Then, −25 kV reversed polarity was applied and the electrophoretic current was monitored until it reached approximately 95% of its original value. At this point (93 s), the polarity was switched back to +25 kV for 6 min and the separation was completed. Under the selected conditions the current was 90 μA.

Under reversed polarity, the cations and neutrals exit the capillary into the waste buffer reservoir. However, if the electrophoretic current is not carefully monitored, anionic analyte may be lost leading to irreproducible results.

Triplicate injections of the solutions were performed and average corrected peak areas (CPAs) (area/migration time) were used for the quantitation.

In order to equilibrate the system, the data generated from the first two injections in a sequence was not used.

3. Results and discussion

3.1. Preliminary experiments

In a previous paper [21], SDS was selected as micellar additive. A phosphate buffer (Na_2HPO_4–Na_3PO_4) at pH 12.3 was chosen in our study for the following reasons: (i) at this pH it has a high buffer capacity ($pK_{a3}=12$) and (ii) the two compounds showed only negative charges with a very good resolution between the two peaks (both sildenafil and UK have basic functional groups with a pK_a value of 8.8). At pH values around 9.0 ion-pair interactions between the two compounds (positive and negative charges) and the anionic SDS micelles lead to longer migration times and poor resolution.

Fig. 2 shows the electropherograms, with and

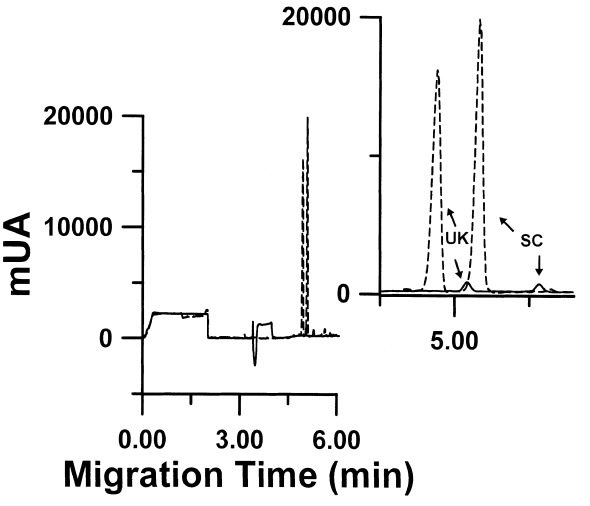


Fig. 2. Electropherograms of a serum spiked with 400 μg/l of UK-103,320 and SC. (a) Operating conditions: 10 m*M* phosphate buffer (Na_2HPO_4–Na_3PO_4), pH 12.3; 30 m*M* SDS, 25 kV, 25 °C, injection time 7 s. (b) Sample stacking with polarity switching (injection time 275 s, polarity reversal time 93 s) 15 m*M* phosphate buffer (Na_2HPO_4–Na_3PO_4), pH 12.3; 25 m*M* SDS, 25 kV, 25 °C.

without stacking, of a serum extract spiked with 400 μg/l of (UK-103,320) and SC. Compared to MEKC alone (injection time 7 s), an injection of 273 s and a polarity switching of 93 s, the determination of SC and UK proved to be more sensitive, yielding signals 11 times larger than those obtained by the direct MEKC proposed by Berzas et al. [21]

3.2. Effect of ionic strength

3.2.1. Sample

A human serum extract spiked with SC (80 μg/l) and UK (80 μg/l) was used to study this effect. The extracted serum samples were dissolved in different phosphate buffers (0.5–10 m*M*) that contained 20% methanol. The best results were obtained with 1 m*M* phosphate buffer. The two compounds were not resolved when the concentration was above 5 m*M*. The injection time was always 100 s and the polarity switching time was calculated in every separation.

3.2.2. Separation electrolyte

The effect of buffer concentration was studied in the 5 to 30 m*M* range with a constant 25 m*M* concentration of SDS showing that when the concentration of the buffer was increased, the migration times of SC and UK also increased. A buffer concentration of 15 m*M* was selected to maintain good peak shape and low current in order to minimize noise and baseline aberrations. Under these conditions, the migration times for UK and SC were 4.80 and 5.08 min, respectively.

3.3. Effect of SDS concentration

Fig. 3 shows the influence of adding SDS to the electrolyte on migration time. Although the SDS concentration has virtually no effect on the velocity of the EOF, it influences the mobility of the two compounds. Since SC and UK are negatively charged at the separation pH, the change observed is due to their hydrophobicity and to an electrostatic repulsive effect with the like-charged micelles. This effect resulted in a slow change in the migration times as a function of SDS concentration for the two compounds. 25 m*M* SDS was selected for further experiments as it gave the best resolution.

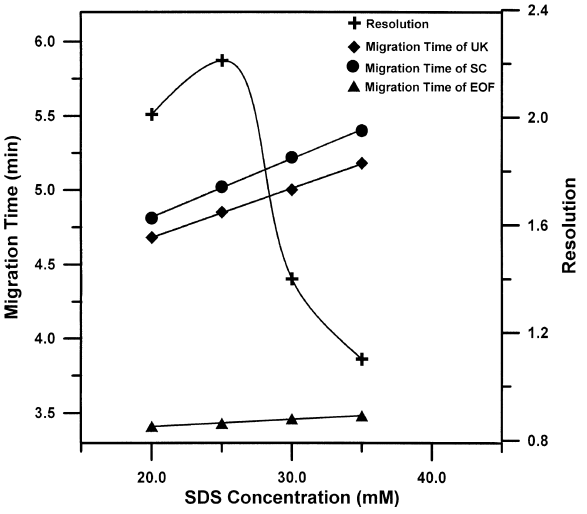


Fig. 3. Influence of SDS concentration on migration times and resolution. Operating conditions: 15 m*M* phosphate buffer (Na_2HPO_4–Na_3PO_4), pH 12.3; 25 kV, 25 °C, injection time 275 s (polarity reversal time 93 s).

3.4. Influence of voltage

The study of this effect was done using a human serum extract spiked with SC (80 μg/l) and UK (80 μg/l) in 1 m*M* of phosphate buffer and 20% methanol. The effect of varying the voltage from 5 to 30 kV was investigated using the same experimental conditions as above. Every experiment was done with a 100 s of injection time and different switching time (95% of maximum current). A potential of 25 kV, in both steps, yielded the best compromise in terms of run time, current generated and efficiency of separation. This potential was used in subsequent stages of method development.

3.5. Optimisation of injection times

In order to improve the detection limits in human serum, injection time has been optimised. With this technique, several hundred-fold increase in injected sample amount can be achieved using a sample plug as long as two-thirds of the total column length. The expression derived by Burgi and Chien [3] was used to calculate the maximum filled length possible without loss of analyte (l_{max}). This calculation is based on the difference of mobility between the analyte and the EOF yielding l_{max}=14.2 cm and 125

s of injection time (28% of total capillary length using a capillary of 60 cm total length×75 μm I.D. and 0.5 p.s.i. injection pressure).

In practice, however, it is easier to fill the whole capillary with the sample solution using different injection times and perform sample stacking. Injection time was varied between 100 and 300 s always using a pressure of 0.5 p.s.i. The corrected peak area increased with longer injection times. Values above 275 s also lead to poor resolution between SC and UK. For this reason, the optimum value chosen was 275 s.

3.6. Optimisation of stacking time with polarity switching

Long stacking time can lead to loss of analyte from the capillary start stacking times give inadequate focusing and poor resolution. To find the moment when the sample buffer was almost completely removed from the capillary, the current was monitored and compared with the current for a column completely filled with the separation buffer. First, current decreases during injection. As the sample buffer is pushed out of the column, the current increases again. When it reaches about 95% of the current of the pure separation buffer, the polarity is switched again, and separation can occur. In our case, 93 s was the selected time for the polarity switching step after 275 s of injection time.

3.7. Performance evaluation

3.7.1. Specificity

The specificity of the method versus endogenous components of the matrix were assessed using three different pools of human serum.

Table 2
Theoretical and experimental values for F_1 and F_2 obtained in the ANOVA test

	$F_{1(exp.)}$	$F_{1(theor.)}$	$F_{2(exp.)}$	$F_{2(theor.)}$
UK	1.83	3.00	3.25	3.49
SC	1.71	3.00	2.39	3.49

3.7.2. Linearity of the response

In all cases, the results were calculated using migration time CPAs. Linearity was assessed by injecting solutions of SC and UK in extracted serum in the 82–900 μg/l concentration range (n=5) for a constant injection time (275 s) and a constant reverse polarity time (93 s).

The RSD values between the slopes were 3.50% (UK) and 4.86% (SC). The results of linearity experiments carried out on the same day confirm the intra-day precision.

The results of linearity experiments carried out on four different days confirm that inter-day precision is still acceptable for biological applications. Individual linear regression equations were calculated for each of the four linearity experiments (each one at five concentrations). Variance analysis showed that each graph was linear and went through or was very close to the origin.

The linearity of the response was evaluated in four successive days at five levels of concentration for each analyte. The analysis of variance (ANOVA) of regression was carried out to compare the different regression lines in order to see if the data can be combined to estimate the appropriate parameters of an overall regression line [22,23]. The analysis of variance values are shown in Tables 1 and 2. The experimental values of F_1 and F_2 are always lower than the theoretical. Therefore, two different cali-

Table 1
The analysis of variance of regression

Source of variation	Sums of squares		Degrees of freedom		Mean squares	
	UK	SC	UK	SC	UK	SC
Deviation within each set from the set line	80.134	181.693	12	12	6.678	15.141
Differences among the regression coefficients	65.114	108.713	3	3	21.705	36.237
Deviation between set lines	73.387	155.401	6	6	12.231	25.900
Total deviations from overall linear regression	153.522	337.094	18	18	8.529	18.727

bration graphs can be proposed for UK and SC, respectively.

The RSD values between the slopes were 5.39% (UK) and 4.37% (SC). The RSD values are acceptable for biological applications and show the ruggedness of the proposed method.

The RSDs calculated at each concentration of UK and SC (Table 3) also show an acceptable intra-day precision for drug monitoring in human serum. The intra-day precision at each concentration of SC and UK was always better than 8.7%.

3.7.3. Limits of detection (LODs) and quantification (LOQs)

The baseline noise was evaluated by recording the detector response over a period of about 10 times the peak width. LOD was obtained as the sample concentration that caused a peak with a height threefold the baseline noise level. LOQ was calculated as 10-fold the baseline noise level yielding LODs at about 17.0 ng/ml and LOQs at about 56.1 ng/ml for both UK and SC.

Finally, a capillary with a bubble in the detection window was used to reduce the LODs and LOQs. New LODs and LOQs were calculated to be about 8.0 and 26.4 ng/ml for the two compounds.

3.8. Recovery study

Spiked serum samples were used to determine the recovery of UK and SC, in the 200–880 mg/l concentration range. Excellent recoveries were obtained (90–100%) for both compounds (SC and UK). In all cases, triplicate samples were used with RSD values lower than 6%.

Table 3
Intra-day (day 1) mean and RSD values of CPA for UK and SC at the different concentrations

	Concentration (mg/l)	Mean CPA (n=5)	SD	RSD (%)
UK	82	619.00	53.65	8.67
	238	1800.75	30.16	1.67
	386	2850.25	62.49	2.19
	503	3671.75	132.74	3.62
	614	4674.50	254.76	5.45
SC	82	594.75	39.50	6.64
	238	1664.00	46.53	2.80
	386	2636.25	107.92	4.09
	503	3472.50	33.43	0.96
	614	4191.50	207.97	4.96

4. Conclusion

In this work, a method is described for the extraction and determination of sildenafil citrate and its metabolite UK-103,320 in human serum by MEKC coupled with sample stacking and polarity switching.

Recovery, precision, sensitivity and linearity were satisfactory in the 80–900 μg/l range, better than those obtained by the methods proposed in the literature. The developed method allows the determination of SC and UK with an LOD of 17 ng/ml for both compounds in the extracted human serum.

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