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# Development of a capillary gas chromatographic method with flame ionisation detection for the simultaneous determination of sildenafil and its *N*-demethylated metabolite in biological fluids

A simple, rapid, and sensitive method has been developed for the analysis of sildenafil (S) and its *N*-demethylated metabolite UK-103,320 (UK) using capillary gas chromatography with a flame ionisation detector. Optimal conditions were investigated, achieving analysis times shorter than 12 minutes. Aspects such as stability of the solutions, linearity, accuracy (>91%), precision, specificity, and limits of detection (0.02 mg/L and 0.12 mg/L for S and UK respectively) were tested in order to validate the method. A solid-phase extraction (SPE) prior to the analysis allows quantification of S in serum and urine, and UK in serum at clinically relevant concentrations. Furthermore the specificity has been assessed by means of mass spectrometry in SCAN mode.

**Key Words:** Sildenafil; UK-103,320; Capillary gas chromatography; Biological sample analysis; Mass spectrometry; Flame ionisation detection

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## 1 Introduction

Sildenafil, chemically designated as 1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-*d*]pyrimidin-5-yl)-4-ethoxyphenyl]sulfonyl]-4-methylpiperazine, is the active ingredient of Viagra. In the pharmaceutical preparations it is present in the form of its citrate salt (**Figure 1**). Sildenafil is a potent and selective inhibitor of cyclic guanosine monophosphate (cGMP) specific phosphodiesterase type 5 (PDE 5) [1–3]. Being capable of enhancing the relaxation of the penile corpus cavernosum, it has the potential to improve penile erectile function.

S is eliminated by hepatic metabolism, the active principal metabolite UK-103,320 (1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-*d*]pyrimidin-5-yl)-4-ethoxyphenyl]sulfonyl]piperazine) (Figure 1) being formed by *N*-demethylation. Both sildenafil and the metabolite have terminal half-lives of about 4 hours, with maximum observed plasma concentrations (about 400 μg/L) being reached within 30 to 120 minutes (median 60 minutes) after oral dosage in the fasted state [1].

After both oral and intravenous administration, sildenafil is excreted as metabolites predominantly in the faeces

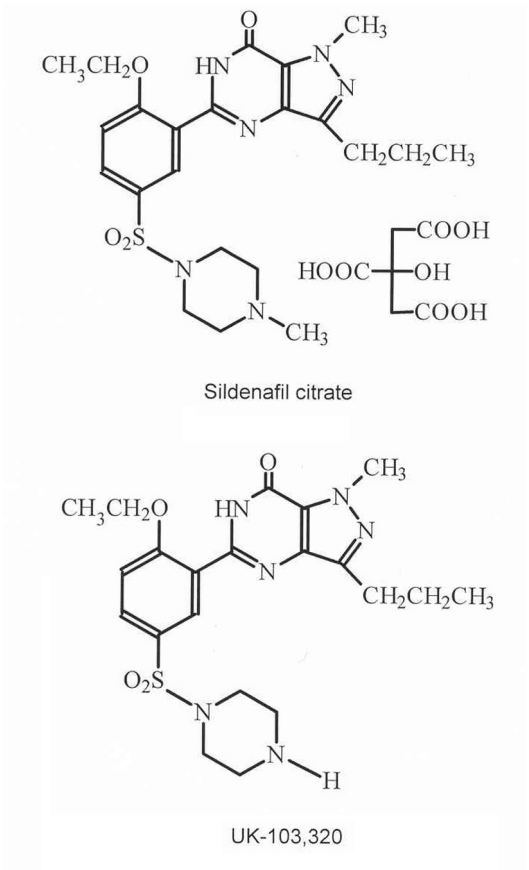


**Figure 1.** Structures of Sildenafil citrate (Viagra) and its metabolite UK-103,320.

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Short Communication

(approximately 80% of an orally administered dose) and to a lesser extent in the urine (approximately 13% of an orally administered dose) [4].

The majority of studies carried out on sildenafil focus on its pharmacokinetic and metabolic behaviour [5–9]. There are few methods reported in literature for its analysis and quantification. The first one was an isocratic HPLC method [10] with spectrophotometric detection for the simultaneous determination of S and its metabolite using automated sequential trace enrichment of dialysates. This HPLC method is still being used by Pfizer laboratories for routine analysis of S in pharmaceutical products.

The determination of S has also recently been achieved in pharmaceutical formulations by adsorptive stripping voltammetry [11].

Berzas et al. [12] have developed a new micellar electrokinetic capillary electrophoresis method for the simultaneous determination of S and its metabolite in human serum.

Recently, new HPLC methods [13,14] have been developed for the analysis of S in plasma samples.

To date, GC has not been used for the analysis of S and UK. Their determination by GC seems difficult without derivatization, but we have developed a simple, rapid, sensitive, and reproducible GC method to measure S in human serum and urine and UK in serum after an extraction and preconcentration process in a $C_{18}$ cartridge and without previous derivatization.

## 2 Experimental

### 2.1 Reagents

Methanol (HPLC grade) was purchased from PANREAC (Madrid, Spain). Sildenafil and its metabolite were supplied as a gift from Pfizer. Stock solutions (126 mg/L and 203.84 mg/L for S and UK respectively) were prepared in methanol and stored in a refrigerator at 4°C.

Solutions used for the study were:

– Standard solutions: prepared daily by dilution of the stock solutions in methanol (0.7–3.5 mg/L).

– Biological standard samples (serum or urine): prepared by addition of S or/and UK (0.07–1.05 mg/L for each compound) to 2 mL biological sample.

### 2.2 Instrumentation

The equipment used was a Hewlett-Packard (Palo Alto, CA) 5890 series II GC provided with a 6890 autosampler, a split-splitless injector, flame ionisation and series 5971 mass-selective detectors with HPG1701AA MS Chemstation software [15].

The column was an HP-5 (5% phenyl-methylsilicone, 15 m × 0.25 mm ID, 0.25 μm film thickness) supplied by Hewlett-Packard.

Direct mass spectra were obtained by means of a VG AutoSpec. magnetic sector mass spectrometer.

Reverse phase $C_{18}$ cartridge (Waters Sep-Pak Plus, Milford, MA, USA).

### 2.3 Treatment of the biological samples

Fresh human blood and urine samples were obtained from different male volunteers.

The serum from the blood samples was kept frozen at –18°C and thawed just before the extraction process.

Fresh urine samples were submitted directly to solid-phase extraction after a preliminary centrifugation step (5000 rev/min, 15 min, 20°C).

### 2.4 Operating conditions

#### 2.4.1 Gas chromatographic procedure

The parameters were as follows: carrier gas: helium; whole flow: 50 mL/min; column head pressure: 110 kPa; flow rate: 1.0 mL/min; injector temperature: 325°C; FID temperature: 300°C; injected volume: 2 μL; oven temperature programme: 60°C (initial column temperature) maintained for 0.75 min (splitless step) and then increased at 70 °/min to 320°C (final column temperature, maintained for 10 min).

Duplicate injections of the solutions were performed and the mean of the absolute peak areas was used for quantification.

Although the main part of the method was developed with flame ionisation detection, the specificity was also assessed using a selective mass detector working in SCAN mode.

For these assays, the general instrumental conditions for MS detection were as follows: interface temperature: 300°C; electronic impact (EI) ionisation energy: 70 eV; EM voltage: 1800 V. Specific conditions for SCAN mode: mass range: 35–550 amu; Scan rate: 1.49 scans/s; and solvent delay: 3.0 min.

#### 2.4.2 Extraction and preconcentration procedure in biological samples

The extraction of S and UK from serum and urine was performed in $C_{18}$ cartridges conditioned before use by means

of 5 mL of methanol followed by 5 mL of 10 mM phosphate buffer pH 7.0 ($NaH_2PO_4/Na_2HPO_4$).

Variable amounts of S and UK were added to the biological samples, and they were then slowly loaded onto the conditioned cartridge. The cartridge was subsequently washed with 8 mL of phosphate buffer and finally S and UK were eluted with 1.0 mL of methanol (spiked biological samples). A volume of 2 μL of this methanol extract was immediately injected for GC analysis.

## 3 Results and discussion

### 3.1 Optimisation of the capillary gas chromatographic procedure

The carrier gas velocity was varied between 15 cm/s (20 kPa head pressure) and 104 cm/s (180 kPa), selecting 68 cm/s (110 kPa) as optimum because it provided maximal efficiency for both compounds; higher velocities led to shorter analysis times but there was also a significant increase in the baseline. The selected head pressure led to a gas flow through the column of 1.0 mL/min at 320°C.

The temperature in the injection port must be maintained high enough to assure volatilisation and to avoid condensation of the samples. The temperature of the injection port was therefore varied between 300 and 375°C, showing increasing peak areas with an increase of this parameter, though this was associated with an increase in baseline aberrations; 325°C was consequently chosen.

Temperature at the top of the column is kept 10–20°C below the boiling point of the solvent, leading to the condensation of the low-volatility components and the solvent. Care must be taken to prevent injector overloading, since this can be a source of contamination in subsequent analyses; the parameters to be optimised are therefore the time and the temperature at which the splitless step takes place.

The splitless time during which the focussing step occurs was varied between 0.1 and 1.20 min, and the value of 0.75 min was selected as optimum at an injector temperature of 60°C the FID temperature of 300°C.

Aliquots of 2 μL were injected and the following oven temperature programme was started.

Using the previously selected injection and detection conditions, several assays were carried out in order to find a temperature programme which provided the quantitative separation of Sildenafil and its metabolite. The best option was the following programme, as it supplied a resolution between peaks ($Rs$) $\geqq$ 2.8 with an analysis time less than 12 minutes.

1) 60°C (Initial column temperature, 0.75 min) splitless step
2) 70 °/min → 320°C (final column temperature, 10 min)

**Figure 2** shows the chromatograms of the two compounds in spiked a) serum and b) urine samples.

### 3.2 Quantitative analytical methodology

#### 3.2.1 Stability of solutions

The stability of three different solutions, standard and spiked biological samples (serum and urine) of S and its metabolite (UK) was determined by comparing the response factors (concentration/average peak areas) of freshly prepared duplicate solutions with those of duplicate solutions stored at room temperature and at 4°C, in darkness and in light. A concentration difference less than 0.2% was found between the freshly prepared diluted solutions and the standard and spiked biological samples stored for 7 days and 1 day. Standard and spiked biological samples were frozen for just one day, their response (after thawing) compared with that of freshly prepared duplicate solutions; no differences were found between them. Stock standard solutions were also tested and found to be stable for a period of at least 3 months.

#### 3.2.2 Linearity

The linearity was assessed by adding different amounts of S and UK to serum and urine samples and subjecting these samples to the analytical procedure. The linear regression equations, the concentration ranges, and the coefficients of correlation are presented in **Table 1**, except for UK in urine samples because of the lack of linearity.

In addition linearity was demonstrated by analysis of variance [16] with $F_{cal} > 4000$ ($P << 0.01$) in all cases.

#### 3.2.3 Accuracy

Several aliquots of S and UK were added to human urine and serum samples. These samples were analysed and good recoveries were obtained for both S and UK in the serum analysis (**Table 2**). However, with urine samples, excellent recoveries were obtained for S, but poor recoveries for UK; in some urine extracts the UK peak did not even appear.

Recoveries were calculated against external standards of lower and upper concentration injected in sequence for every sample.

#### 3.2.4 Precision

Ten injections of one standard solution containing 0.7 mg/L for S and 1.0 mg/L for UK were carried out se-

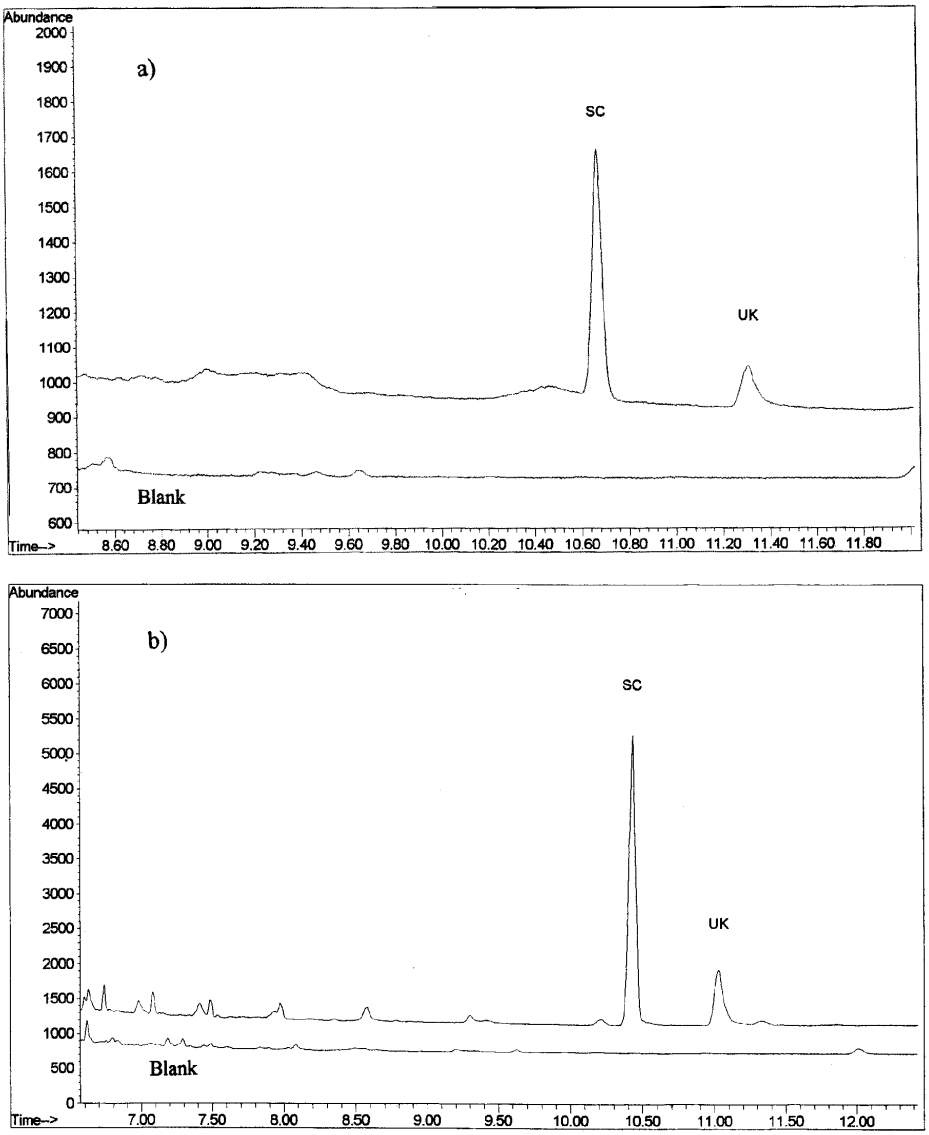


**Figure 2.** FID chromatograms obtained for a mixture of S (0.7 mg/L) and UK (1.0 mg/L) in two different extracts from spiked a) serum and b) urine samples respectively and their respective blank extracts.

**Table 1.** Linearity, range of concentration, LODs, and LOQs for S and UK analyses in biological fluids.

| | | S | UK |
|---|---|---|---|
| Equation[a)] | Serum | $y = (17633 \pm 3600)\,x + (2161 \pm 1500)$ | $y = (3620 \pm 1070)\,x + (410 \pm 416)$ |
| | Urine | $y = (19571 \pm 1000)\,x + (1380 \pm 545)$ | – |
| Coeff. of correlation | Serum | 0.9950 | 0.9950 |
| | Urine | 0.9998 | – |
| Linear range (mg/L) | Serum | 0.07–1.05 | 0.10–1.50 |
| | Urine | 0.10–1.05 | – |
| LODs (mg/L) | | 0.02 | 0.12 |
| LOQs (mg/L) | | 0.06 | 0.36 |

[a)] Concentration ($x$) versus absolute peak areas ($y$).

**Table 2.** Recoveries from spiked serum and urine samples preparation.

| | | S | | | UK | | |
|---|---|---|---|---|---|---|---|
| | | Added (mg/L) | Found (mg/L) | % Recov. | Added (mg/L) | Found (mg/L) | % Recov |
| Serum | Sample 1 | 0.475 | 0.456 | 96 | 0.683 | 0.625 | 91 |
| | Sample 2 | 0.191 | 0.188 | 98 | 0.275 | 0.261 | 95 |
| | Sample 3 | 0.128 | 0.120 | 94 | 0.184 | 0.167 | 91 |
| | Sample 4 | 0.110 | 0.102 | 93 | 0.157 | 0.144 | 92 |
| Urine | Sample 1 | 0.770 | 0.733 | 95 | 1.106 | 0.206 | 19 |
| | Sample 2 | 0.385 | 0.386 | 100 | 0.553 | 0.215 | 39 |
| | Sample 3 | 0.256 | 0.275 | 107 | 0.367 | 0.229 | 62 |
| | Sample 4 | 0.220 | 0.207 | 94 | 0.316 | 0.041 | 13 |

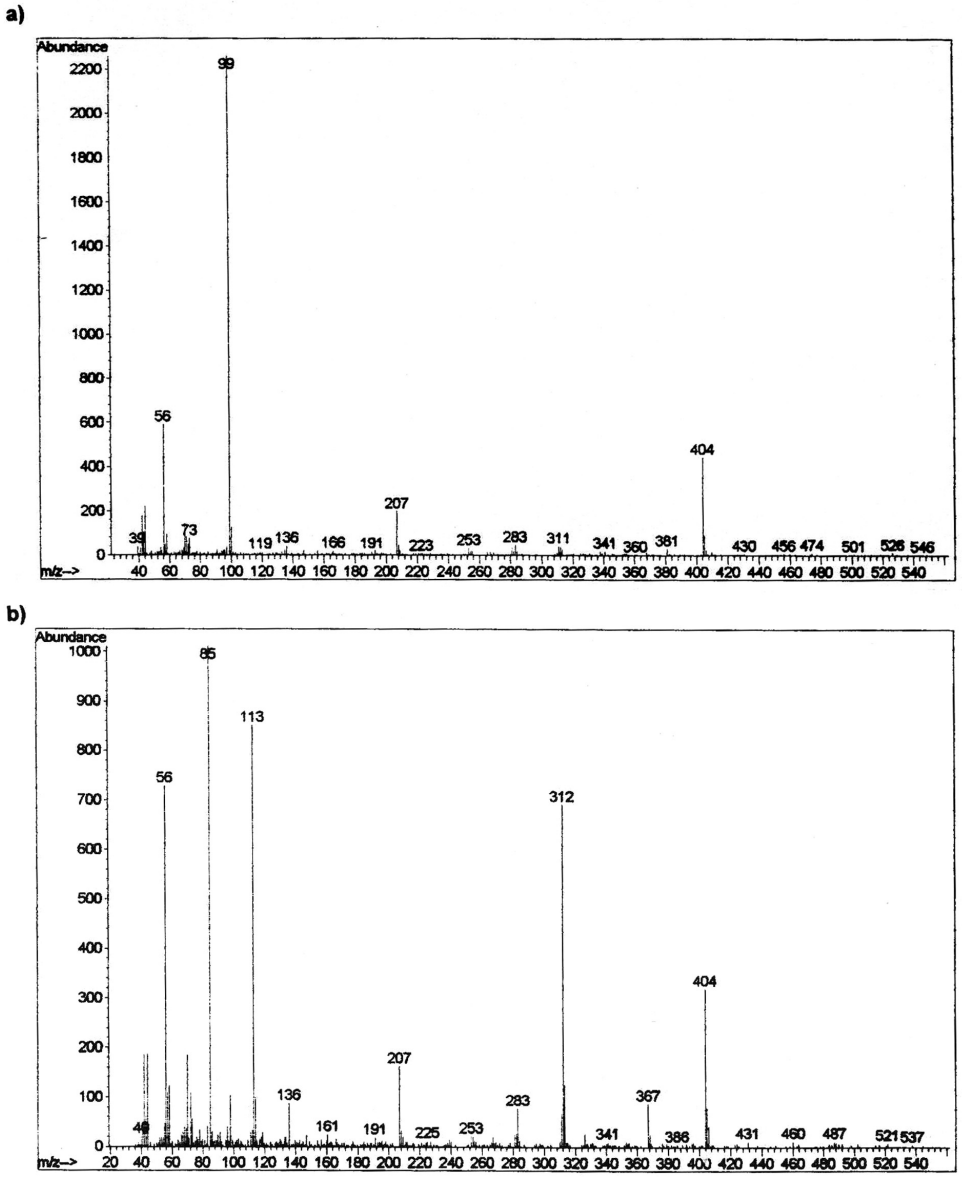


**Figure 3.** GC-mass spectra of a) sildenafil and b) UK-103, 320 standards obtained with an EI system and an ionisation energy of 70 eV.

quentially. The precision of elution times and absolute peak areas showed relative standard deviations of 0.78 and 0.64 for elution times and 1.21 and 2.04 for absolute peak areas for S and UK respectively.

The precision of the overall process (extraction, preconcentration, and GC steps) was evaluated by the analysis at two levels of concentration (0.3 and 0.6 mg/L for UK and 0.2 and 0.4 for S) of spiked serum samples ($n = 3$) from different male volunteers. Recoveries ranging between 92.2 ± 1.9% and 96.6 ± 2.0% for both concentration levels and for the two compounds were found.

### 3.2.5 Specificity

Seven serum and urine samples were subjected to the extraction and preconcentration procedure (without spiking S and UK), and 2 μL of the obtained extract was injected into the GC equipment; no peak was found at the analytes' retention times (Figure 2). As examples, the chromatograms corresponding to the extracts from spiked serum and urine samples and their respective blanks extracts are shown in Figure 2.a and Figure 2.b, respectively.

The peak purity was also checked by using an MS detector in SCAN mode (**Figure 3**).

### 3.2.6 Limits of detection (LODs) and quantification (LOQs)

The LODs and LOQs were calculated by measuring ten blanks using the maximal sensitivity allowed by the system and calculating the standard deviation (SD) of this response. The LOD was estimated by multiplying the SD by a factor of 3 whereas the LOQ was defined as 10 times the SD (Table 1).

## 4 Conclusions

A simple, rapid, and sensitive method has been developed for the simultaneous analysis of S and its metabolite UK in serum and for measuring S in urine using, for the first time, capillary gas chromatography. Prior to GC analysis, samples are concentrated by solid phase extraction which permits quantification of both compounds at clinically relevant concentrations using a flame ionisation detector. Furthermore, the use of a mass selective detector in SCAN mode achieves reliable identification of these compounds in the biological samples and demonstrates the absence of problems due to coelution and artefacts. This approach provides a wider scope of application because of the possibility of establishing a general method for the analysis of S and UK in different biological samples.

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