High Performance Liquid Chromatography Method to Detect Tramadol and Sildenafil in the Blood of Rats on Combination Treatment.

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Abstract

Tramadol is one of the widest abused street drug in Egypt. Recently, drug abusers were taking tramadol beside sildenafil for sexual purposes. So it was important to develop a new HPLC method for the simultaneous determination of tramadol and sildenafil in the blood of rats exposed to a combination of specific dose of tramadol and sildenafil. Sample preparation involved liquid–liquid extraction with methyl tertiary butyl ether (MTBE) and backextraction with hydrochloric acid. Tramadol, sildenafil, and the internal standard nalbuphine were separated by reversed phase HPLC using 60% acetonitrile and 40% 20mM sodium phosphate buffer pH 7.5. Detection was by using UV at wavelengths 230 nm. The method was linear for tramadol (0.8–100µg/ml) and sildenafil (0.3–100 µg/ml) with mean recoveries of 97.2% and 100.8%, respectively. Intra- and inter-day precisions were 9.34% and 11.82%, and 9.12% and 10.43% for tramadol and sildenafil at the respective limits of quantitation (0.8 and 0.3µg/ml). Accuracy for tramadol and sildenafil ranged from 96.6% to 97.8% and 99.2% to 101.3% respectively. The method was applied to a pharmacokinetic study of tramadol and sildenafil in rats.

Key words: hplc, Tramadol, Sildenafil.
Introduction

Tramadol hydrochloride, (±)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl) cyclohexanol (Fig. 1a), is a centrally-acting analgesic used in the treatment of mild to moderate pain (1). Its therapeutic plasma concentration range is 100–300 ng/ml (2). Tramadol is rapidly and almost completely absorbed after oral administration but its absolute bioavailability is only 65–70% due to first-pass metabolism (3). The metabolism of tramadol in human is mediated by cytochrome P4502D6 (CYP2D6) to O-desmethyltramadol (ODT) and N-desmethy tramadol (NDT). ODT is pharmaceutically active and contributes to the analgesic efficacy of tramadol (4).

Sildenafil (1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidine-5-yl)-4-methoxysulfonyl]4-methyl piperazine (fig. 1b) is a potent and selective inhibitor of cGMP-specific phosphodiesterase capable of enhancing the relaxation of the penile corpus cavernosum and therefore having the potential improve penile erectile function. Quantification of sildenafil is essential during the evaluation of drug. Several HPLC methods have been developed for determination of sildenafil in various samples, such as dietary supplements (5-7), pharmaceutical preparation (8), and mouse skin (9), human plasma and urine (10,11,12). For a review see “Sildenafil determination in various matrices” (13) Analyses were based on liquid-liquid extraction at basic pH (6, 7). For this purpose we used this simple, rapid and accurate HPLC method.

Experimental

Chemicals and reagents

Pure tramadol hydrochloride was obtained from Minapharm, Cairo, Egypt, Sildenafil citrate was obtained from Pfizer pharmaceutical company (Pfizer Ltd., Egypt), and nalbuphine was obtained from Amoun Pharmaceutical Co. El-Obour City (Cairo, Egypt). Acetonitrile, Methanol and methyl tertiary butyl ether (MTBE) (HPLC grade) were purchased from Merck (Darmstadt, Germany). Deionized water was produced by a Milli-Q Millipore Water System (Milford, MA). Potassium dihydrogen phosphate and sodium tetraborate decahydrate were from J.T. Baker (Deventer, Holland). All other reagents and materials were of analytical grade and supplied from commercial sources. The aqueous and organic
components of the mobile phase, degassed under pressure, were mixed by the HPLC. The LC mobile phases were filtered through 0.2-mm cellulose acetate membrane filters (Sartorius Stedim Biotech S.A.; Aubagne Cedex, France) with a solvent filtration apparatus.

**Standard solutions**

Stock solutions of Tramadol, sildenafil and the internal standard nalbuphine were prepared to a concentration of 1,000 µg/mL in MeOH using volumetric flasks. These were then stored at −20°C. To obtain final concentration of 100 µg/mL, appropriate dilutions of stock standard solutions were prepared by diluting 1 mL of each solution to 10 mL. These solutions of Tramadol, sildenafil, and the internal standard nalbuphine were serially diluted in glass tubes (10 mL) to reach final concentrations of 5, 1, 0.5 and 0.1 µg/mL. Then they were stored at −20°C.

**Instrumentation and chromatographic conditions**

HPLC was performed with an Agilent (USA) series 1100 Quadrupump and Diode Array DETECTION. C18 5µm Eclipse × DB column with particle size 5µm (150×4.6mm) was used. The mobile phase consisted of 60% acetonitrile (A)–40% 20 Mm KH2PO4 buffer (B) adjusted to pH 7.5 with NaOH at a flow rate of 1.5 mL/min.

**Animal treatment and sampling**

Animal experiments were conducted at the animal experimental facility of the national research center (Cairo-Egypt). Twenty-four Wistar breed rats weighing 130 to 150 g were used for the experiment. The rats were randomly divided into eight groups consisting of three animals. These rats were housed two per cage, under conventional ventilation, temperature (18-20°C) and lighting (16 h light/12 h dark) conditions. During the study, they were given free access to water and food. The health of the rats was monitored daily by qualified personnel supervised by a veterinarian for the duration of the study. The animals were given one week to adjust to their new environment before commencement of the experiment. During this adjustment period, all animals were kept on drug-free feed. After the adjustment period, Group GI received an single injection of saline while the other groups received a combination of Tramadol at 100 mg/kg and Sildenafil at 100 mg/kg; all injections were given orally. The animal groups were sacrificed after 30 min (GI), 1 h (GII), 2 (GIV), 4 h (GV), 6 h (GVII), 9 h (GVIII) and 24 h (GIVIII), respectively, following administration.

The animals were decapitated and the blood samples collected immediately. All samples were immediately frozen at −20°C until analysis.

**Sample extraction**

The procedure was performed in a 15-mL screw-capped polypropylene vial. A 1-mL aliquot of whole blood was added to 300 µL of IS (100 µg/mL). After vortexing for 30 s, 0.5 mL of Na2B4O7 10H2O buffer (0.2 M, pH 9.0) was added and the sample was vortexed again. Six mL of MTBE was then added, shaken (60 osc/min, 10 min) and centrifuged at 2,191 g (rotor radius 10 cm) for 10 min. Five mL of the supernatant was collected in a 15 mL screw-capped polypropylene vial containing 1 mL of 0.01 M HCl. This latter blend was vortexed (30 s), shaken (60 osc/min, 5 min) and centrifuged at 2,191 g (rotor radius 10 cm) for 5 min. The organic layer was discarded. The aqueous layer was alkalized with 0.5 mL of 0.2 M borate buffer and the mixture was extracted with 5 mL of MTBE by shaking for 5 min followed by centrifugation at 2,191 g (rotor radius 10 cm) for 3 min. Four mL of organic layer was transferred to a 5-mL glass tube for complete evaporation in speed vacuum concentrator. The residue was reconstituted with 50 µL of an amobile phase of acetonitrile–Phosphate buffer (10:90) and 100 µL was injected onto the HPLC.

**Bioanalytical method validation**

The described method was validated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, specificity, stability, precision and accuracy according to international guidelines on the bioanalytical method validation (14). Calibration curves were obtained by spiking the blank matrix with a known concentration of each drug and internal standard to provide concentrations of 2.5, 5, 10, 30, 50, and 70 µg/mL. The calibration curves of peak area versus concentration (µg/mL) of the investigated drugs were plotted. Least squares regression parameters for the calibration curves were calculated, and the concentrations of the test samples were interpolated from the regression parameters. Sample concentrations were determined by linear regression, using the formula $Y = mX + b$, where $Y$ peak area, $X$ concentration of the standard in mg/mL, $m$ the slope of the curve, and $b$ the intercept with $Y$ axis. Correlation coefficients for each of the calibration curves were: 0.997 (within-run and between-run accuracy and precision were reassessed on quality control samples (QC samples) and determined by replicate analysis using seven determinations of different concentration levels: LOD (0.8 µg/mL; 0.3 µg/mL for Tramadol and sildenafil), low QC (2.5 and 5 µg/mL), medium QC (10 and 30 µg/mL) and high QC (100 µg/mL).

**Quantification**

When unknown samples were assayed, a control and a fortified blank sample were processed simultaneously for quality control. LODs and LOQs were determined as analyte concentration giving signal-to-noise ratios of 3 and 10, respectively.
Statistical analysis and pharmacokinetic analysis

The statistical analyses were evaluated using an analysis of variance (ANOVA) test. The results were presented as means ± standard deviation (SD). All the analyses were conducted using GraphPad InStat (GraphPad Software; La Jolla, CA). For all the experiments, differences were considered significant if the associated probability level (P) was lower than 0.05. The pharmacokinetic calculations were carried out using WinNonLin v 5.2.1 (Pharsight Corp.; Sunnyvale, CA). Maximum concentration (Cmax) for all the investigated drugs in blood and the time required to reach Cmax (Tmax) were predicted from the data. Changes in blood concentrations for all the investigated drugs were evaluated using the standard non-compartmental analysis and the relative pharmacokinetic parameters were determined using standard non-compartmental equations.

Results and Discussion

Detection method development

The mobile phase was chosen on the basis of a previously published method. The Phosphate buffer was tested at different concentrations (0.01, 0.02, 0.05 and 0.1M). At the lower concentration, Tr and IS resulted in the same retention time. The investigated drugs were well separated from blood impurities at buffer concentrations of 0.02, 0.05 and 0.1M, and 0.02M was chosen as optimal because higher concentrations can cause salt precipitation in the HPLC. A range of buffer pH (3.0 to 8) was assayed to optimize the chromatographic separation. Optimal peak separation for pure investigated drugs was produced using a pH ranging between 7 and 8, but the actual working is pH 7.5. The final mobile phase resulted in acetonitrile–NaH2PO4 (0.02M), pH 7.5 with a 1.5 mL/min flow rate. This was found to be an excellent compromise in terms of sensitivity and peak separation. The wavelengths tested in the present study were: 275 nm (15) ,225 nm (16) and 290 nm (17). The wavelength value of 230 nm was found to be optimal in terms of sensitivity for all the investigated drugs and avoiding several matrix impurities that became problematic at lower wavelengths.

The internal standard was chosen based on previous studies on Tramadol.

Optimization of the extraction conditions

The influence of the kind of solvents (an important tool for the selectivity of the method) was studied to find the optimal extraction protocol for the investigated drugs. The solvents ethylacetate (20,18), MTBE (19) and chloroform (commonly used in the Forensic Toxicology Lab; Cairo, Egypt) were compared. MTBE was selected as the most suitable organic solvent in terms of analyte extraction and minimization of matrix interference (Table I). Optimal pH value for extraction was 9.

Table I

| Organic solvents | Tramadol | sildenafil | IS |
|-----------------|----------|------------|----|
| Ethylacetate    | 85.1±5.4 | 76.5±6.4   | 25.2±2.4 |
| Chloroform      | 80.4±4.4 | 45.3±3.7   | 81.1±5.2 |
| MTBE            | 97.2±6.39| 100.8±5.33| 99.8±3.5 |

Method validation

It was found that there were no published methodologies for simultaneous determination of Tramadol, Sildenafil and Intraanal standard from rat blood samples using HPLC–UV,so it was necessary to validate each step of the suggested analytical method. The calibration curves were constructed by plotting the ratioof the peak areas versus concentrations in the working range. Good linearity was achieved for Tramadol and sildenafil in the range studied. The linear regression equations are reported in Table II. According to Europea Medicine Evaluation Agency (EMEA) guidelines (14), LODs and LOQs were calculated based on a signal-to-noise approach. These calculations were performed by comparing measured signals from samples with known low concentrations of investigated drugs with those of blank samples. In this way, the minimum concentration at which the investigated drugs can be reliably quantified (LOQ) or detected (LOD) was determined. The typical signal-to-noise ratios were 10:1 and 3:1 for LOQ and LOD, respectively (Table III). Both the accuracy and the precision of these values lay within the proposed criteria [relative standard deviation (RSD), 20%]. Specificity and interference by co-eluting components were determined by comparing the chromatograms of different batches of blank matrices to those from spiked whole blood and test samples. It was found that under optimized chromatographic conditions, peaks due to the matrix did not interfere with Tramadol, sildenafil and IS. Typical retention times for Tramadol, sildenafil and IS were 2.8±0.2, 1.7±0.05 and 2.2±0.05 min, respectively (Figures 2A,2B and 2C). Recoveries were 97.2±6.39 % for Tramadol, 100.8±5.33% for sildenafil and 99.8±3.5% for IS. The relative coefficient of variation (CV) (%) values varied from 3.48 to 9.34, 5.33 to 11.82, and 1.06 to 4.41 for Tramadol, sildenafil and IS, respectively. Intra-day accuracy consistency (repeatability) was evaluated for five replicates of each QC sample during the same day. Inter-day accuracy consistency (immediate precision) was evaluated by quantization of Tramadol, sildenafil and IS in QC samples on five different days. Relative errors for both the intra-day and inter-day accuracy were 7%. Stability studies were performed.
to ensure good reproducibility of the method. Stock solutions of the investigated drugs and IS (10 µg/mL) and high and low QC samples were tested for stability under short-term room temperature conditions, long-term storage conditions (−20°C) and freeze-thaw treatment. Tramadol, sildenafil, and IS were very stable at both 20°C for 24 h and −20°C for 30 days. Data obtained after three freeze-thaw cycles showed that the investigated drugs were stable in rat blood (CV, 7%). These findings indicated that the storage of investigated drugs in blood samples at −20°C is adequate, and no stability-related problems would be expected during routine analyses for analytical studies within 10 days. Robustness of the methodology was determined by the reproducibility of results using the (analytical) method in 20°C; a loss of 10–12% was observed and different laboratories or under different circumstances. The present study evaluated three blood aliquots from the treated rats in two different labs (Faculty of science, Al-Azhar University, and Chemistry Lab., Forensic Medicine Authority, Cairo, Egypt) and obtained variations of less than 7.2%. These results demonstrate that the method enables accurate quantification of Tramadol and sildenafil. The validation parameters were in agreement with the EMEA guidelines (14). Although to the best of our knowledge, no simultaneous detection of Tramadol and sildenafil in rat blood is present in the literature, the present findings are in line with previous HPLC studies (critical in forensic medicine), taking into consideration the singular investigated drugs (17, 21, 22, 23).

Table II Validation Data for Tramadol and sildenafil

| Parameter               | Tramadol     | sildenafil   |
|-------------------------|--------------|--------------|
| Linear range (µg/mL)    | 0.8-100      | 0.3-100      |
| Calibration equation    | y = -0.0087 + 0.0269 x | y = 0.778 + 0.0649 x |
| Correlation coefficient (r²) | 0.997       | 0.997       |
| Recovery (%)            | 97.2±6.39    | 100.8±5.33   |
| LOQ (µg/mL)             | 0.8          | 0.3          |
| LOD (µg/mL)             | 0.3          | 0.1          |
| Accuracy (%)            | 96.6-97.8    | 99.2-101.3   |
| Precision (%)           |              |              |
| Intra-day               | 3.48-9.34    | 5.33-11.82   |
| Inter-day               | 3.8-9.12     | 5.41-10.43   |
| Specificity             | Specific     | Specific     |

**Application of the method**

The applicability of this method was verified by determining Tramadol and Sildenafil in rat blood samples after oral administration of a combination of Tramadol and Sildenafil. HPLC analysis of the blood confirmed the presence of Tramadol and Sildenafil in time-related amounts (Figure 3). The amount of Tramadol and Sildenafil in blood ranged between 0.38 and 12.31 µg/mL and 2.3 and 9.55 µg/mL, respectively. The described method allowed the pharmacokinetics of the four investigated drugs to be followed. Tramadol and Sildenafil had a Cmax after 1 h. Tmax of Tramadol and Sildenafil are also in line with a recent pharmacokinetic study on rats (24). For this reason and others, because the present data have been derived using only two rats for each collection time, a large-scale study is indicated to fully determine the significance of the results. This method could also be applied to the detection of Tramadol and Sildenafil in pharmaceutical preparations.
(Fig 2 a): chromatographic curve from pure substances and IS (25 µg/mL).

(Fig 2 b): chromatographic curve from fortified sample (2, 5 µg/mL) of Wistar rat blood
(Fig 2 c): chromatographic curve from blood sample collected from Wistar rat orally injected with a combination of tramadol (100 mg/kg) and sildenafil (100 mg/kg) (collection at 60 min).

(Fig 3a) Scatterplot of Con. ug/ml blood vs Time (h)
**Conclusion**

The described analytical method provides selective and accurate determination of Tramadol and Sildenafil without the need for expensive cleanup steps, solvent-consuming flows or expensive devices. The LOQs are within acceptable limits which is matched with therapeutic does and show that the method could be useful for forensic toxicological analysis on abusers.

These features also make the described method suitable for pharmacokinetic investigations, including drug–drug interaction. In summary, this is the first time that HPLC–UV technique has been reported to simultaneously detect of Tramadol and Sildenafil. This method (extraction, separation and applied techniques) is simple precise, accurate, and efficacious for the determination of investigated drugs in rat blood and pharmaceutical preparations.

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