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Stability of fluoxetine in stored plasma, aqueous, and methanolic solutions determined by HPLC with UV detection

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The stability of fluoxetine was studied in plasma, and in aqueous and methanolic solutions at a concentration of 6 μg/ml under different storage temperatures and time intervals up to three months. Fluoxetine exhibited good stability at −20 and 5 °C, but was unstable at room temperature under the same conditions. A significant loss was observed at the second, third and fifth weeks in plasma, aqueous solution and methanolic solution respectively ($P > 0.95$). At the end of the experiment, the amount of fluoxetine-recovered was at least 55.25% regardless of the storage conditions. Chromatography was performed using a C8 column and the mobile phase consisted of methanol/acetonitrile/triethylamine solution (35 : 20 : 45) adjusted to pH 5.5. UV detection was at 230 nm. Fluoxetine was isolated from plasma by liquid-liquid extraction (LLE) with dichloromethane as extracting solvent after addition of 20% ammonia solution. The standard curve was linear over the range of 0.05−10 μg/ml. The inter-day coefficient of variation and the lower detectable limit were 6.92% and 0.05 μg/ml respectively.

1. Introduction

Fluoxetine (FLX) is a selective serotonin reuptake inhibitor antidepressant drug [1]. TheFDA approved the drug in 1987 for the treatment of depression and in 1994 for the treatment of obsessive compulsive disorder. The half-life of FLX is 2−3 days, and about 60% of an oral dose are excreted in urine and 12% in feces. Peak plasma concentrations are reache in 6−8 h with effective steady-state plasma concentrations ranging from 0.3 to 0.5 μg/ml, after 2−4 weeks [2]. Several gas chromatographic (GC) methods have been developed for the determination of FLX [3−5]; howevers, the necessity of a derivatization step is time consuming and introduces potential sources of error [6]. Other HPLC methods involve extensive sample preparation with a lengthy back extraction procedure [7−9] or using a complicated precolumn derivatization procedure [10, 11] while yet other methods have been inadequately validated [12].

FLX has been associated with many fatality cases [13−15]; it is therefore necessary to study its stability in order to get meaningful interpretations of laboratory results. Unfortunately, there are limited data regarding the stability of drugs of forensic and clinical interest in biological fluids, especially when those samples may be stored under different conditions and subject to a variable degree of putrefaction for days or even weeks between specimen acquisition and drug quantitation [16−19]. There are no data available regarding the stability of FLX, and so this article presents a study on the effect of different storage temperatures on the stability of FLX in plasma, and aqueous, and methanolic solutions using a new simple HPLC method.

2. Investigations, results and discussion

2.1. HPLC-UV method

The mobile phase developed consisted of methanol/acetonitrile/0.2% triethylamine solution (35 : 20 : 45), adjusted to pH 5.5 with glacial acetic acid. This mobile phase was able to separate FLX and the internal standard diazepam efficiently with symmetrical sharp peaks and acceptable retention times of 6.11 min and 9.73 min for FLX and internal standard respectively (Fig. 1). The standard curve was linear ($r^2 = 0.9996$), over a range of 0.05 to 10 μg/ml. The drug was extracted with dichloromethane after adjusting the pH with 20% ammonia solution. The recovery was 70% and 87% for FLX and the internal standard diazepam respectively. The organic layer was evaporated to dryness, reconstituted with mobile phase and injected into the HPLC system. The intra-day and inter-day coefficients of variation of six determinations at a concentration of 5 μg/ml were 4.73% and 6.92% respectively. The lowest drug concentration that could be detected significantly by the HPLC method developed was 50 ng/ml with a signal of at least three times more the background noise. Possible in-

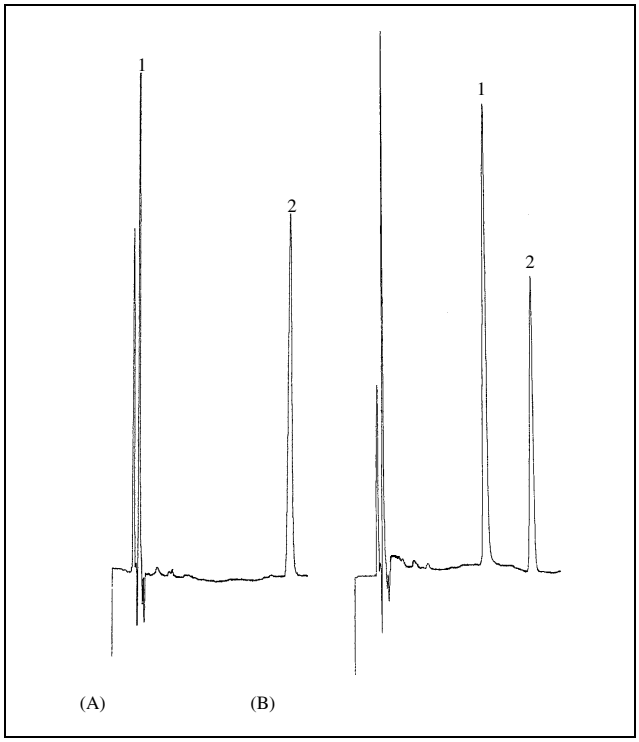

Fig. 1: A: Chromatogram of blank plasma with (1) solvent front, (2) 1 μg/ml i.s., B: Chromatogram of plasma spiked with (1) 5 μg/ml FLX and (2) 1 μg/ml i.s.

Table: Retention times in the drug interference study

Tested drugs	Retention time (min)	Tested drugs	Retention time (min)
Fluoxetine	6.11	Diazepam	9.73
Amoxapine	12.35	Promethazine	8.37
Clomipramine	11.56	Bromazepam	8.11
Haloperidol	9.89	Midazolam	2.10
Phenobarbital	5.85	Alprazolam	5.23
Carbamazepine	9.12	Paracetamol	3.25
Amitriptyline	17.50	Imipramine	>22
Thiopental	13.14	Chlordiazepoxide	14.25

HPLC, C8, using the mobile phase developed Acetonitrile: Methanol: TEA solution (20 : 35 : 45)

terference from drugs frequently administered to psychiatric patients was investigated using the same analytical conditions (Table); no interference was observed.

2.2. Stability of FLX in plasma samples

Fig. 2 shows the decline in the amount of FLX recovered from plasma samples stored at 5 °C, room temperature and −20 °C. Plasma FLX samples analysed after one week showed no significant decrease in concentration with recoveries of above 89.6% regardless of the storage temperature. The first significant decrease in plasma FLX concentration stored at room temperature was observed after two weeks of storage with a measured recovery of 77.6% with P value > 0.95, however, no significant loss was observed for plasma samples stored at 5 and −20 °C. At the end of the experiment, FLX recoveries ranged from 55.3% to 90.4% depending on the storage temperature.

2.3. Stability of FLX in aqueous, and methanolic solution

The results of analysis are presented in Fig. 3 and 4 for aqueous and methanolic solutions respectively. The aqueous solution of FLX analysed after two weeks showed no significant decrease in concentration with recoveries of above 92.2% regardless of the storage temperature. The first significant decrease in aqueous FLX concentration stored at room temperature was observed after three weeks of storage with a measured recovery of 85.1%. The methanolic solution of FLX analysed after four weeks showed no significant decrease in concentration with recoveries of above 94.2% regardless of the storage temperature. The first significant decrease in methanolic FLX concentration for storage at room temperature was observed after five weeks of storage with a measured recovery of 91.7% with

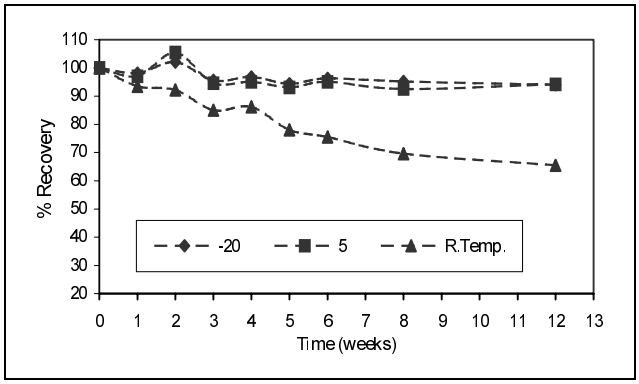

Fig. 3: Changes in FLX recovery with time in aqueous solution

P > 0.95. However, no significant loss was observed for either aqueous or methanolic samples stored at 5 and −20 °C. At the end of the experiment, FLX recoveries ranged from 65.5% to 94.3% and 77.7% to 94.1% for aqueous and methanolic solutions respectively, depending on the storage temperatures.

In conclusion, the HPLC and LLE methods developed are suitable for clinical, forensic and pharmaceutical quantitation and separation of FLX. Due to a variation in the time from sample collection to analysis or repeated analysis, factors affecting the analyte in the sample should be considered before interpreting the results as storage conditions could seriously affect the level of the analyte. Factors which lead to these changes are temperature [16], storage time, bacterial contamination, putrefaction [20], the analyte medium, pH and light [21]. In this experiment temperature and time factors were studied such as to mimic the conditions under which real samples are kept. FLX showed better stability in methanol compared with plasma and aqueous samples stored at the same temperatures for the same period of time. Samples stored at −20 °C and 5 °C showed the greatest apparent stability of FLX. There are large numbers of possible reactions leading to drug degradation and most may be classified as either hydrolysis or oxidation. In hydrolytic reactions temperature, pH and the presence of water are the major factors that influence drug decomposition, while oxidation reactions are strongly influenced by environmental factors such as light and metal ions [22]. On the other hand, one of the main criteria for potential destruction of a compound during the putrefaction process appears to be the presence of oxygen in its chemical structure [23]. Unfortunately, a trace amount of oxygen was sufficient to produce a significant stability problem [24]. The FLX structure has an ether aryl structure with an oxygen atom, which acts as a bridge

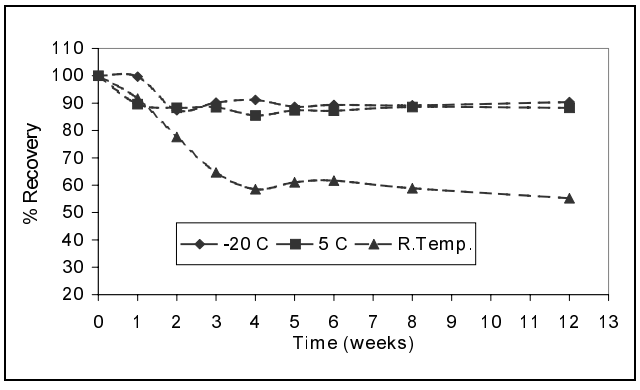

Fig. 2: Changes in FLX recovery with time in plasma

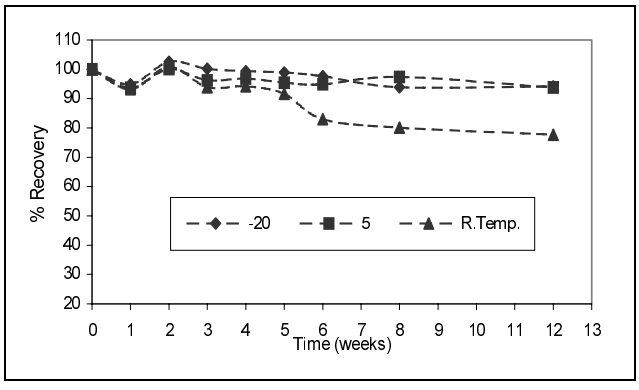

Fig. 4: Changes in FLX recovery with time in methanolic solution

that links the two starting materials. The oxygen in the ether, like that in alcohols, may be protonated to generate an alkyl/aryl oxonium ion [25]. The reactivity of these ions may lead to ether cleavage, with p-trifluoromethylphenol and α-[2(methylamino) ethyl] benzene methanol considered to be the major degradation products, although *N*-methyl-3-phenyl-2-propen-1-amine also arises either as a minor degradation product or as an impurity of FLX [26, 27].

3. Experimental

3.1. Reagents

The drug standards FLX (Lilly), and diazepam (Roche) were donated by the drug quality control laboratory, Ministry of Health, Ammans Jordan. The solvents dichloromethane (Janssen Chimica, Belgium), acetonitrile and methanol (Lab-Scan Ltd. Dublin, Ireland) used were HPLC grade solvents. Ammonia, triethylamine (Sigma-Aldrich Company Ltd., England), glacial acetic acid (Lab-Scan Ltd. Dublin, Ireland) and other chemical reagents were of analytical grade. The extraction tubes, graduated pipettes, micropipettes, volumetric flasks, 12×75 mm culture tubes and other glassware were available at the toxicology laboratory. The fresh plasma was obtained from the blood bank of the Jordan University Hospital.

3.2. Apparatus and chromatographic conditions

The HPLC system used for drug analysis consisted of a HP 1100 isocratic pump equipped with a HP 1050 UV spectrophotometer detector (Hewlett-Packard, CA, USA), HP 3395 integrator (Hewlett-Packard, CA, USA) and Rheodyne injector valve with a fixed 20 µl loop (Cotati, CA, USA). The analytical column used was a C−8 Luna (250 mm × 4.6 mm I.D.) with 5 µm particle size, purchased from Phenomenex, USA. The mobile phase developed consisted of methanol/acetonitrile/0.2% triethylamine solution, (35 : 20 : 45) adjusted to pH 5.5 with glacial acetic acid. The mobile phase was introduced into the HPLC system at a flow rate of 1.8 ml/min, the effluent of the column was constantly monitored at 230 nm.

3.3. Extraction procedure

In a 10-ml glass tube, one ml of 20% ammonia solution was added to 1 ml of sample (plasma, aqueous and methanolic solutions) and 100 µl of 10 µg/ml internal standard (I.S.). Dichloromethane (5 ml) was added to the above mixture as an extraction solvent. Extraction was performed on a rotary shaker for 15 min. After centrifugation (10 min, 5,000 × g), the organic layer was transferred to a 12×75 mm culture tube and evaporated to dryness under a gentle stream of nitrogen at 40 °C. The residue was dissolved in 100 µl of mobile phase before being injected into the HPLC system.

3.4. Design of stability study and statistical analysis

The stability of FLX was studied by storage of spiked plasma samples, aqueous and methanolic solutions at different temperatures, then analysing them at intervals up to three months. The spiked plasma samples of FLX were prepared as 6 µg/ml, which represents the toxic and fatal level in biological fluids [13−15]. Freshly prepared samples of plasma, aqueous and methanolic solutions of the drugs were used to calculate the concentration of the drugs in the spiked samples. The quantity of drug recovered initially (day zero) was taken as 100%. Successive determinations was made and the "% of the original present" was calculated for each drug. The results were considered to be statistically significant if the reduction in the recovery of the drug in question exceted 1.96 of the coefficient of variation corresponding to a probability (P value) >0.95 [28, 29].

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