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Assay validation for three antidepressants in pharmaceutical formulations: Practical approach using capillary gas chromatography

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Abstract

An easy and fast capillary gas chromatographic FID method, which was already described by the same authors for the simultaneous determination of fluoxetine, fluvoxamine and clomipramine without derivatization step, is now submitted to a validation procedure in several pharmaceutical formulations. Main aspects of the validation method are examined and discussed, since methods for regulatory submission in most cases must demonstrate: specificity in presence of all potential components, concentration range over which the response is lineal, accuracy, precision, acceptable detection and quantitation limits and stability of the procedure. The pharmaceutical preparations subject of validation were: 'Prozac' (capsules), 'Dumirox' (tablets) and 'Anafranil' (tablets) containing fluoxetine, fluvoxamine and clomipramine, respectively. The results presented in this report show the applied gas chromatographic method is acceptable for the determination of the three antidepressants in the pharmaceutical formulations above mentioned.
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1. Introduction

The use of selective serotonin reuptake inhibitors (SSRIs) is widely prescribed in therapy for depression, obssesive–compulsive disorder, panic attack disorder, bulimia, social phobia, and post-traumatic stress disorder [1–7]. SSRIs are non-tricyclic antidepressants that enhance serotoninergic neurotrasmission process, though selective inhibition of neuronal reuptake of serotonine in presynaptic neurons. The chronic inhibition of serotonine reuptake leads to downregulation of serotoninergic 5-HT_1 presynaptic inhibitory autoreceptors and to increase serotonine release.

In general terms, SSRIs have received widespread popularity in everyday clinical practice and are preferred with regard to classic tricyclic antidepressants. SSRIs exhibit few side effects in terms of frequency and severity. The major problem, as for other antidepressants, is their great inter-individual variability in clinical response, which makes it difficult to evaluate the correct posology.

Fluoxetine, fluvoxamine are two SSRIs drugs that enhance serotoninergic neurotransmission through the selective inhibition of neuronal reuptake of serotonin [8] and clomipramine, a tricyclic ternary amine that has been applied for the therapy of depression and obsessive–compulsive disorders.

In this work, an easy and fast capillary gas chromatographic method, previously proposed by the authors for the simultaneous determination of fluoxetine, fluvoxamine and clomipramine [9] without previous derivatization step, is submitted to a validation procedure in three different pharmaceutical formulations, according with official validation guidelines for bioanalytical applications in the pharmaceutical industry.

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Method validation is the process of proving that an analytical method is acceptable for its intended purpose. In pharmaceutical industry, validation of analytical method is required in support of product registration applications [10]. For pharmaceutical methods, guidelines from the United States Pharmacopeia [11], International Conference on Harmonisation [12] and the Food and Drug Administration [13,14] provide a framework to perform such validations.

Many of the principles, procedures and requirements of validation are common to the majority of analytical methods. Validation is performing by conducting a series of experiments using the specific conditions of the method and the same type of matrix as the intended samples. It entails evaluation of various parameters of the method such as accuracy, precision (reproducibility), linearity (concentration-detector response relationship), sensitivity, limits of detection and quantitation, recovery from the matrix and specificity (selectivity). The definitions and procedures used to calculate these parameters are adequately described in many publications related to pharmaceutical [15–23] and biomedical [24–30].

The aim of this work is to validate the gas chromatographic method above mentioned on the following three pharmaceutical preparations: prozac (capsules) containing fluoxetine and excipients, dumirox (tablets) containing fluvoxamine and excipients and anafranil (tablets) containing clomipramine and excipients. This method could be a valuable alternative to the existing official methods established by the European Pharmacopeia [31].

2. Experimental

2.1. Reagents

Methanol (HPLC grade) was purchased from PANREAC.

Fluoxetine clorhidrate, fluvoxamine maleate and clomipramine clorhidrate were purchased from TOCRIS Coolson LTD. and distributed by BIOGEN CIENTÍFICA S.L.

Placebos of pharmaceutical formulations of fluoxetine were purchased from ACOFARMA, those ones of fluvoxamine were from SOLVAY PHARMA company and the same ones of clomipramine were from NOVARTIS FARMACEUTICA.

Standard solutions (200 mg/L) were prepared in methanol and stored in the refrigerator at 4 °C. Working standard solutions were daily prepared by diluting the stock standard solutions with methanol.

2.2. Instruments

The used equipment was: a Hewlett–Packard 5980 Series II GC (Palo Alto, CA) provided with a 6890 autosampler, a split/splitless injector, flame ionization and 5971 Series mass selective detectors and HPG1701AA MS Chemstation software [32].

The column was a HP-5 (5% phenyl–methylsilicone, 15 m × 0.25 mm i.d., 0.25 μm film thickness) adquired from Hewlett–Packard.

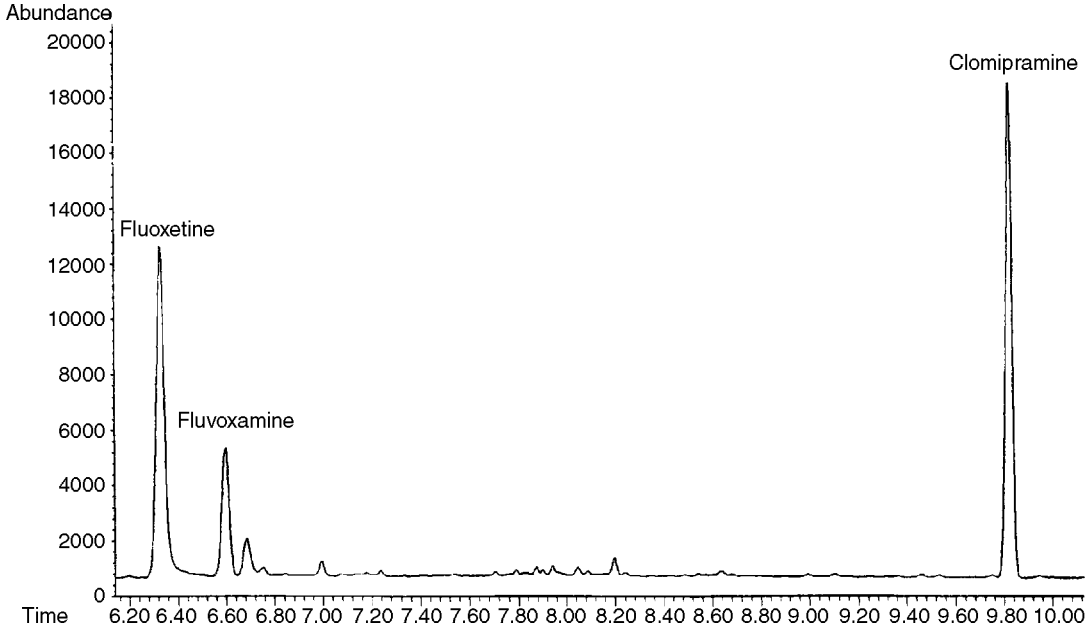

Fig. 1. Capillary gas chromatogram for a standard mixture of fluoxetine, fluvoxamine and clomipramine in the following experimental conditions, carrier gas: helium, total flow: 50 mL/min, head pressure column: 80 kPa, flow rate: 1.2 mL/min, injector temperature: 260 °C, FID temperature: 250 °C, injected volume: 2 μL, oven temperature program: 60 °C kept for 0.75 min, then programmed at 70 °C/min (held for 0.5 min), at 1 °C/min to 185 °C (held for 0.5 min) and at 70 °C/min to 250 °C (held there for 5 min).

2.3. Operating conditions

The gas chromatographic method subject of validation was:

Carrier gas: helium, total flow: 50 mL/min, head pressure column: 80 kPa, flow rate: 1.2 mL/min, injector temperature: 260 °C, FID temperature: 250 °C, injected volume: 2 μL, oven temperature program: 60 °C kept for 0.75 min, then programmed at 70 °C/min to 182 °C (held for 0.5 min), at 1 °C/min to 185 °C (held for 0.5 min) and at 70 °C/min to 250 °C (held there for 5 min).

In Fig. 1, it is shown the obtained chromatogram for a standard mixture of the three antidepressants in these operating conditions.

Since all the pharmaceutical preparations analysed only contain one of the studied antidepressants, any of the other two drugs could be used as internal standard to achieve quantitation following "internal normalization criterion" [33] in these pharmaceutical applications. So, in the validation procedure developed for fluoxetine in prozac capsules, clomipramine was used as internal standard, whereas in the same way, for validation procedures of fluvoxamine and clomipramine in dumirox and anafranil tablets, clomipramine and fluoxetine were respectively used as internal standards.

Duplicated injections of the solutions were performed and average relative peak areas were used for the quantitation, using in all the analysis a content of 10 mg/L of the antidepressants selected as internal standard in each quantitation.

2.4. Pharmaceutical formulations

- Prozac (20 mg capsules, Eli Lilly S.A.) containing fluoxetine clorhidrate, starch of maize and dimethilcone.
- Dumirox (100 mg tablets, Duphar, S.A.) containing fluvoxamine maleate, manithol, starch of maize, sodium estearilfumarate, pregelatinized starch, silica coloidal anhidre, methylhydroxipropylcelulose, polyethylenglycol 6000, talcum powder and titanium dioxide.
- Anafranil (75 mg tablets, Novartis Farma S.A.) containing clomipramine clorhidrate, silicic coloidal acid, calcium phosphate dibasic, calcium estearate, hydroxipropylmethylcelulose, red iron oxide, castor oil, talcum and titanium dioxide.

2.5. Solutions

Duplicated test and standard solutions were prepared as follows:

2.5.1. Standard solutions

Weigh accurately about 20 mg of fluoxetine (clorhidrate), or fluvoxamine (maleate) or clomipramine (clorhidrate), dissolve in methanol shaking by means of a magnetic stirrer for 5 min, transfer to 100-mL calibrated flask and dilute with methanol to the mark. From these stock solutions, take measured aliquots and dilute with methanol in appropriate calibrated flasks to give different final contents of fluoxetine or fluvoxamine or clomipramine.

2.5.2. Test solutions

2.5.2.1. Fluoxetine (capsules prozac). Six capsules were emptied, weighed accurately and the contents were mixed thoroughly. A quantity of the power equivalent to 20 mg of fluoxetine was dissolved in about 70 mL of methanol and shaked mechanically for 5 min. The suspension was transferred into a 100-mL calibrated flask and diluted with methanol to the mark. After a centrifugation step, an aliquot (500 μL) from the supernatant was diluted 1/20 (v/v) with methanol to give a final concentration of about 10 mg/L of fluoxetine, also adding a known amount of stock solution of clomipramine to obtain a content of 10 mg/L (internal standard).

2.5.2.2. Fluvoxamine (tablets dumirox). Six tablets were weighed and ground in a mortar. A quantity of the power equivalent to 100 mg of fluvoxamine was transferred into a beaker and about 100 mL of methanol were added, it was mechanically shaking for 5 min. The suspension was transferred into a 500-mL calibrated flask and diluted with methanol to the mark. An aliquot from the supernatant (500 μL) was diluted with methanol 1/20 (v/v) to give a final concentration about 10 mg/L of fluvoxamine, also adding a known amount of stock solution of clomipramine (10 mg/L) to quantify fluvoxamine.

2.5.2.3. Clomipramine (tablets anafranil). The procedure was the same as above described for fluvoxamine tablets, but in this case, the final concentration prepared for clomipramine was 15 mg/L, adding a known amount of fluoxetine (10 mg/L) as internal standard.

2.5.3. Analytical placebo

The analytical placebo stock solutions were prepared taking into account the amount specified by pharmaceutical companies. In all cases, these stock solutions contain all the components indicated in the pharmaceutical formulation except the corresponding antidepressant.

2.6. Validation of the proposed method

Method validation entails evaluation of the following parameters on the pharmaceutical formulations before cited:

2.6.1. Stability of solutions

2.6.1.1. Standard solutions. The stability of standard solutions of fluoxetine, fluvoxamine and clomipramine was determined by comparing the response factors (concentration/average peak area) of duplicated solutions stored at room temperature and 4 °C, in the dark and in the light, with those ones of freshly prepared duplicated solutions.

2.6.1.2. Test solutions. The stability of test solutions was assessed by comparing the fluoxetine, fluvoxamine and clomipramine content of a capsule or tablets stored at room temperature for 24 h with those of a freshly prepared standard solutions.

2.6.2. Specificity

Peak purity was checked for fluoxetine, fluvoxamine and clomipramine in their pharmaceutical formulations by the use a MS detector in SCAN mode. Analysis of peak purity were performed by means of a HPG 1701AA Chemstation software (32).

For these assays, the instrumental MSD conditions were: interface temperature: 280 °C, ionization energy: 70 eV, EM voltage: 1800 V, mass range: 35–350 amu, scan rate: 2.30 scans/s and solvent delay: 2.5 min.

2.6.3. Linearity and accuracy studies

The linearity and accuracy of the analytical procedure was assessed by recoveries studies for the three drugs in a range between 50 and 150% ($n = 5$) of the targeted working concentration, which were added to an amount of 200 mg of matrix (analytical placebo) placed in calibrated flasks. Two independent determinations were performed for each analysis. So, from this experiment it was obtained the relationship between the analytical signals (relative areas) versus the added amount and also the accuracy by means of a recovery study.

2.6.4. Precision

The precision of the test validation procedure was assessed spiking each specific matrix with an amount of fluoxetine or fluvoxamine or clomipramine standards corresponding to 100% of theoretical content and it was independently analysed eight times ($n = 24$, 3 days). This assay was achieved by two operators.

Furthermore, six capsules (to determine fluoxetine) or six tablets (to determine fluvoxamine or clomipramine) were separately analysed.

In all cases, quantitation was made taking into account relative peak areas.

2.6.5. Limits of detection (LOD) and quantitation (LOQ)

The LOD and LOQ were calculated by measuring ten specific placebo solutions for each antidepressant, using the maximal sensitivity provided by the system and calculating the standard deviation (S.D.) of this signal. LOD and LOQ were calculated using flame ionization detector (FID).

3. Results and discussion

3.1. Stability of solutions

The response factors of standard solutions of fluoxetine, fluvoxamine and clomipramine were found to be unchanged for at least 7 days as much stored at room temperature as at 4 °C, in the dark or in the light. Less than a 0.2% concentration difference was found between the solution freshly prepared and those aged for 7 days. The solutions can therefore be used during this period without the results being affected. It is obvious that such a long period of time does not normally occur before performing measurements in a control laboratory, but a test every day can be recommended to cover possible instrumental delay.

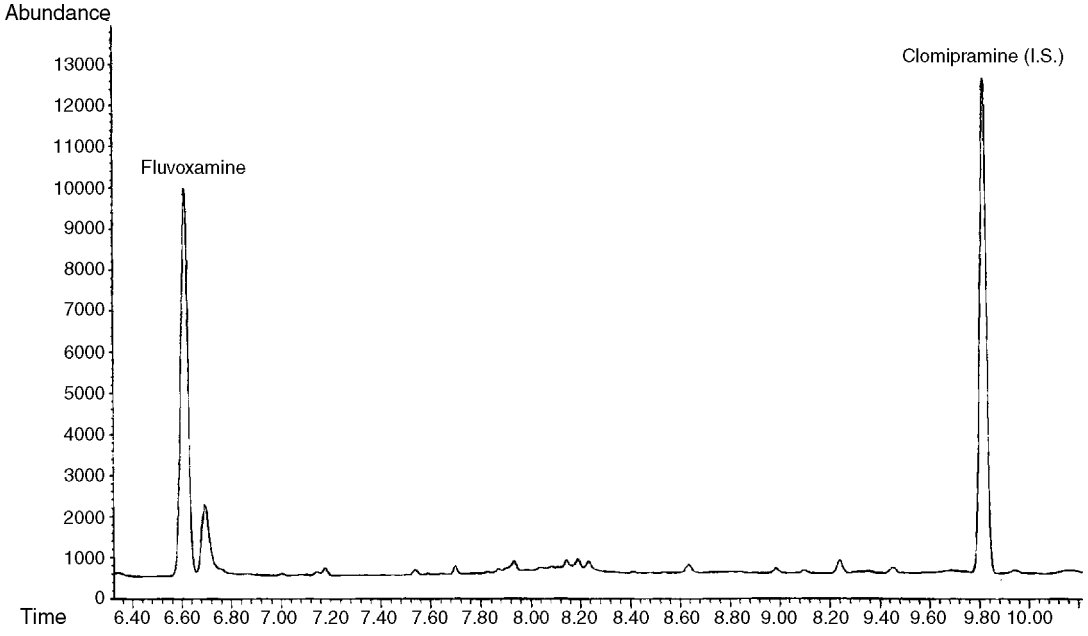

Fig. 2. Capillary gas chromatogram for a sample of dumirox tablets (fluvoxamine) using clomipramine as internal standard.

Test solutions: The stability of test solutions was assessed by comparing the fluoxetine, fluvoxamine and clomipramine content of a capsule or tablets stored at room temperature for 24 h with those of a freshly prepared standard solutions, do not finding significative differences between them.

3.2. Specificity

As in any separation technique, co-elution of peaks is possible in capillary gas chromatography; therefore, it is useful to investigate the purity of separated peaks in test solutions.

As an example, chromatogram obtained for a dumirox tablet is shown in Fig. 2.

Although quantitation of drugs amount in pharmaceutical formulations was achieved using flame ionization detector, peak purity was checked for the analyzed pharmaceutical formulations by the use of a MS detector working in SCAN mode. Analysis of peak purity was performed by means of a HPG 1701AA MS Chemstation software [32].

The evaluation of peak purity by the software is based on "Fragmentography" technique, also called "Mass Chromatography", in which several characteristic ions of the mass spectrum of a compound are selected following criteria like abundance and specificity; the software determine the peak chromatographic symmetry and the maximum of this one, it means that the system determine how would be the chromatographic peak for each one of the selected ions and compare the obtained chromatograms for each one of these selected ions. Taking into account the retention time and the number of the scan at which is obtained the peak maximum of the selected ions for a compound, the system judge the peak purity of a MS chromatographic peak.

No interferences from the excipients of the studied formulations were observed, but it is important to emphasize, that as it can be seen either in Figs. 1 and 2, two consecutive peaks were obtained for fluvoxamine signal in both standard and test compounds. Mass spectra of both consecutive peaks were checked when the peak purity was investigated showing the same mass spectra for both of them, which proved that these two peaks are corresponding to the two different fluvoxamine stereoisomers (E and Z), being the E isomer (the first peak) the main component and the active principle whereas the Z isomer (the second one) appears like an impurity. This fact is in agreement with findings of other workers [34].

3.3. Linearity and accuracy

The linearity of an analytical method can be defined as its ability within a definite range to obtain results directly proportional to the concentration of the analyte in the sample. For assay methods, this study is generally performed by preparing spiked placebo solutions at five concentration levels, from 50 to 150% of the targeted analyte concentration. Five levels at least are required to allow detection of curvature in the plotted data. The 50–150% range for this study is wider that what is required by the FDA guidelines. Solutions should be prepared and analyzed a minimum of two times.

Acceptability of linearity data is often judged by examining the correlation coefficient and *y*-intercept of the linear regression for the response versus concentration plot. A correlation coefficient of >0.999 is generally considered as evidence of acceptable fit of the data to the regression line. The *y*-intercept should be less than a few percent (≤2%) of the response obtained for the analyte at the target level [17].

In our case, in order to study linearity and accuracy of the proposed method, several aliquots of fluoxetine, fluvoxamine and clomipramine corresponding to 50, 75, 100, 125 and 150% of the targeted working concentration were added into their respective analytical placebo (test solutions).

So, the detector response measured for the studied antidepressants was linearly correlated with the concentration of each antidepressants injected. The obtained regression lines, calculated using least-squares method, were:

Fluoxetine:

$$Y = (8.03 \times 10^{-2} \pm 7.0 \times 10^{-2}) + (0.1718 \pm 7.2 \times 10^{-3})X, \quad r^2 = 0.9965, \quad t_{exp} = 1.15, \quad t_{theor} = 2.571$$

Fluvoxamine:

$$Y = (-0.111 \pm 2.8 \times 10^{-2}) + (5.345 \times 10^{-2} \pm 2.9 \times 10^{-3})X, \quad r^2 = 0.9943, \quad t_{exp} = 1.32, \quad t_{theor} = 2.571$$

Clomipramine:

$$Y = (-7.37 \times 10^{-2} \pm 5.2 \times 10^{-2}) + (0.1998 \pm 3.6 \times 10^{-3})X, \quad r^2 = 0.9967, \quad t_{exp} = 1.41, \quad t_{theor} = 2.571$$

where Y = relative peak areas, X = concentration of solutions (mg/L) and r^2 = coefficient of determination.

Confidence intervals were calculated with $P = 0.05$ considering four degrees of freedom. Each point of the calibration graph is corresponding to the main value obtained for three independent area measurements. The satisfactory determination coefficient showed that fluoxetine, fluvoxamine and clomipramine responses were linear over the studied concentration range. The regression lines passed through the origin. These results allow us to use only one concentration of the standard solution in the test procedure.

The accuracy of a method is the closeness of the measured value to the true value for the sample. Accuracy is usually determined in one of the following four ways:

1- First, accuracy can be assessed by analyzing a sample of known concentration and comparing the measured value to the true value. From National Institute of Standards and Technology (NIST) reference standards are often used: however, such a well-characterized sample is usually not available for new drug-related analytes.

2- The second approach is to compare test results from the new method with results from an existing alternative method that is known to be accurate. Again, for pharmaceutical studies, such an alternative method is usually not available.

The third and fourth approaches are based on the recovery of known amounts of analyte spiked into sample matrix.

3- The third approach, which is the most widely used recovery study, is performing by spiking analyte in sample matrix. For general assay methods, spiked samples are prepared in triplicate at three levels over a range of 50–150% of the targeted concentration [24].
4- The fourth approach is the technique of standard additions, which can also be used to determine recoveries of spiked analytes. This approach is used if it is not possible to prepare a blank sample matrix without the presence of the analyte.

In our case, the accuracy of the procedure was assessed using only one concentration of the standard solution (100% of the theoretical content prepared in duplicate) to bracket the measurements of the test solutions. The concentration found in the test solutions are then calculated by reference to the triplicate bracketing standard solutions and the obtained recoveries for each concentration of antidepressants test solutions are shown in Table 1.

The accuracy and the linearity of the procedure over the tested range can also be assesed from the graph of found concentration versus the added concentration. This graph should have a slope of unity and should pass through the origin if the procedure was accurate and linear. The obtained equations were:

Fluoxetine:

$$\text{Found concentration} = (3.96 \times 10^{-2} \pm 5.0 \times 10^{-1}) + (0.9992 \pm 0.017)C_{\text{add}}, \quad r^2 = 0.9996, \quad t_{\text{exp}} = 0.2184$$

Fluvoxamine:

$$\text{Found concentration} = (-0.1333 \pm 5.0 \times 10^{-1}) + (1.024 \pm 0.017)C_{\text{add}}, \quad r^2 = 0.9996, \quad t_{\text{exp}} = 0.7390$$

Clomipramine:

$$\text{Found concentration} = (0.3779 \pm 6.6 \times 10^{-1}) + (0.9717 \pm 0.015)C_{\text{add}}, \quad r^2 = 0.9996, \quad t_{\text{exp}} = 1.5637$$

The slopes of these lines are not significantly different from unity. These lines pass through the origin ($t_{\text{cal}} = 0.22$, 0.74 and 1.56 for FLX, FLV and CLO, respectively). These *t*-values corresponded to $P \gg 0.05$. Therefore, the tested procedure could be considered as accurate and linear in the checked concentration range.

In order to use one standard solutions for calibration, it is necessary that the regression lines obtained from the standard and the test solutions pass through the origin, but also that the slopes of these regression lines are comparable. A significant difference in the slopes could indicate a matrix effect. However, since the method proposed is linear and accurate by considering only one concentration for the standard solution, it can be concluded there is no matrix effect.

3.4. Precision

The precision of an analytical method is the amount of scatter in the results obtained from multiple analyses of a homogeneous sample. To be meaningful, the precision study must be performed using the exact sample and standard preparation procedures that will be used in the final method.

The first type of precision study is instrument precision or injection repeatability. The second type is repeatability or intra-assay precision. The remaining precision study involves much of what historically has been called ruggedness. Intermediate precision is the precision obtained when the assay is performed by multiple analysts, using multiple instruments, on multiple days, in one laboratory. The last type of precision study is reproducibility, which is determined by testing homogeneous samples in multiple laboratories.

In our case, in order to check the precision of the test gas chromatographic procedure, eight injections of standards of fluoxetine, fluvoxamine and clomipramine on their respective analytical placebo solutions were carried out sequentially ($n = 8$). This operation was repeated over 3 days. The precision of the retention times and relative peak areas, in terms of R.S.D. (relative standard deviation) were excellent, since

Table 1
Recoveries

Fluoxetine			Fluvoxamine			Clomipramine		
Added	Found	% Rec.	Added	Found	% Rec.	Added	Found	% Rec.
5.00	5.10 ± 0.02	102.1 ± 0.3	4.97	5.04 ± 0.05	101.5 ± 1.0	7.50	7.60 ± 0.04	101.2 ± 0.5
7.50	7.36 ± 0.06	98.1 ± 0.8	7.34	7.38 ± 0.07	100.5 ± 0.9	11.25	11.49 ± 0.03	102.1 ± 0.3
10.50	10.60 ± 0.22	100.8 ± 2.1	9.94	9.95 ± 0.05	99.7 ± 0.4	15.00	14.75 ± 0.05	98.3 ± 0.4
12.00	12.14 ± 0.05	101.2 ± 0.5	12.31	12.34 ± 0.07	100.1 ± 0.6	18.75	18.73 ± 0.02	99.8 ± 0.1
15.40	15.36 ± 0.08	99.8 ± 0.5	14.91	15.28 ± 0.08	102.5 ± 0.5	22.5	22.20 ± 0.25	98.7 ± 1.1

$n = 3$.

the obtained values were 0.11, 0.11 and 0.08 and for retention times and 4.0, 4.5 and 3.5 for relative peak areas of fluoxetine, fluvoxamine and clomipramine, respectively ($n = 24$).

In order to evaluate the physical and chemical interactions that could take place during the manufacturing process, the previously prepared spiked placebos were analyzed and the found recoveries were 100.37 ± 0.45 for fluoxetine, 98.59 ± 0.58 for fluvoxamine and 100.13 ± 0.32 for clomipramine ($n = 8$).

Furthermore, repeatability studies were carried out to determine fluoxetine in prozac capsules, fluvoxamine in dumirox tablets and clomipramine in anafranil tablets ($n = 6$) upon procedure given in test solutions section. The amount of FLX per capsule and FLV and CLO per tablets was found 20.16 ± 0.55, 101.16 ± 3.23 and 74.60 ± 1.53 mg, respectively.

Intermediate precision of the test procedure can be assesed in different ways. One way is to use different operators (≥ 2), working in different days, under variable conditions (reagents, etc.). Each operator should apply the procedure under conditions of repeatability. The procedure was applied to a placebo spiked with an amount of analyte corresponding to the nominal 100% value ($n = 8$) by two different operators in two different days, to assess the accuracy at the targeted concentration. The average recoveries from a placebo spiked with 100% fluoxetine content were 100.37 ± 0.56 (operator 1), 101.38 ± 0.47 (operator 2), for fluvoxamine assay were 98.59 ± 0.60 (operator 1) and 98.28 ± 0.57 (operator 2) and for clomipramine assay were 100.13 ± 0.55 (operator 1) and 99.92 ± 0.84 (operator 2).

3.5. Limits of detection (LOD) and quantitation (LOQ)

The LOD and LOQ were calculated by measuring ten specific placebo solutions for each antidepressant, using the maximal sensitivity allowed by the system and calculating the standard deviation of this signal.

Under these conditions, the obtained LODs were 10.1, 105.3 and 40.0 μg/L and the found LOQs were 33.5, 300.0 and 80.0 μg/L for FLX, FLV and CLO, respectively. The LOQs were subsequently validated separately by the analysis of six specific spiked placebos approximately prepared at their respective concentrations for fluoxetine, fluvoxamine and clomipramine.

4. Conclusions

This paper has intended to propose a basic experimental design to validate a simple capillary gas chromatographic procedure for the analysis of three active ingredients in their respective pharmaceutical formulations. It has been shown that the experimental results concerning to linearity, accuracy, precision and specificity of the test validation on the three different placebos prove the reliability of the gas chromatographic procedure for its intended application. Therefore, this method has proved to be useful and adequate for the analysis of fluoxetine in prozac capsules, fluvoxamine in dumirox tablets and clomipramine in anafranil tablets and it could be an alternative to traditional existing methods for the determination of the three antidepressants in their pharmaceutical formulations.

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