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*Original Article*

# A simple and sensitive HPLC method for analysis of imipramine in human plasma with UV detection and liquid-liquid extraction: Application in bioequivalence studies

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## *Abstract*

High-performance liquid chromatography (HPLC) methods employing ultraviolet (UV) detector are not sufficiently sensitive to measure the low plasma concentrations following single oral dose of imipramine. Therefore, in the present study a simple, rapid and yet sensitive HPLC method with UV detection was developed and validated for quantitation of imipramine in human plasma samples. An efficient liquid-liquid extraction (LLE) of imipramine from plasma with the mixture of hexane/isoamyl alcohol (98:2) and back extraction of the drug in acidic medium concomitant with evaporation of organic phase allowed the use of UV detector to conveniently measure plasma levels of this compound as low level as 3 ng/ml. Separation was achieved on a µ-Bondapak $C_{18}$ HPLC column using sodium hydrogen phosphate solution (0.01 M)/acetonitrile (60/40 v/v) at pH 3.5 ± 0.1 at 1.5 ml/min. Trimipramine was used as the internal standard for analysis of plasma samples. The retention times for imipramine and trimipramine were 4.3 and 5.2 min, respectively. Calibration curve was linear in the range of 3-40 ng/ml using human plasma with the average extraction recovery of 85 ± 5%. Imipramine was found to be stable in plasma samples with no evidence of degradation during three freeze-thaw cycles and three months storage at -70 ºC. The current validated method was finally applied in bioequivalence studies of two different imipramine products according to a standard two-way crossover design with a two weeks washout period.

***Keywords:*** HPLC; Imipramine; Human plasma; Bioequivalence study

## INTRODUCTION

Tricyclic antidepressant (TCA) drugs have commonly been used to treat endogenous depression, panic attacks, phobic states, neuropathic pain and pediatric enuresis (1,2). The antidepressant effects of TCA drugs are largely due to their ability to inhibit prejunctional re-uptake of norepinephrine and serotonin (1,2). Imipramine is one of the most widely used of this class which is metabolized in the body to desipramine with more potent inhibitory effect of norepinephrine (1–4). Imipramine with low molecular weight is very liposoluble, readily and completely absorbed from gastrointestinal tract with the peak levels occurring at 2-3 h after oral administration. The majority of the dose of the drug is excreted in the urine mainly as metabolite and plasma half-life is 8-20 h (5,6). Different analytical methods have been reported in the literature for the assay of imipramine in biological fluids including spectrofluorimetry (7), isotope-derivative dilution analysis (8), gas-liquid chromatography (9), and high-performance liquid chromatography (HPLC) (10). In recent years, HPLC has been frequently used for the determination of imipramine in biological fluids with either ultraviolet (UV) (10,11), fluorescence (5,12), electrochemical (13-15), diode-array (16,17) or mass spectrometry (MS) detection (18). None of the HPLC methods employing ultraviolet (UV) detector were sufficiently sensitive to measure the low plasma concentrations following single oral dose of

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the drug. Fluorescence and MS detections have provided the most sensitive means of measuring plasma levels of imipramine and its major metabolites, desipramine. However, both procedures involve expensive equipment which are not affordable at most laboratories. In the present method, an efficient liquid-liquid extraction (LLE) of imipramine from plasma allowed the use of UV detector to conveniently measure plasma levels of this compound and extend the sensitivity of the assay to 3 ng/ml.

Many different clean up procedures have been reported so far to recover imipramine from plasma samples including protein precipitation (5,11,15), solid phase extraction (19), and LLE (10,11,14). Although some studies have been performed with regard to HPLC determination of imipramine in plasma after LLE, none of them are appropriately developed and validated to be entirely applicable and reproducible to the pharmacokinetic studies of this drug. Chen and coworkers (14) proposed an HPLC method for determination of imipramine in plasma and urine samples using LLE and UV detection. The calibration curve was linear in the range of 20-500 ng/ml and the method was not validated according to the International Conference on Harmonization (ICH) guidelines. In some other similar studies after LLE of plasma samples, the limit of quantitation (LOQs) of imipramine was reported to be between 15 to 40 ng/ml (10,11). The quantification limits in the aforementioned studies is not sufficiently low to characterize precisely the drug plasma profiles especially at later time points after oral administration of the drug where the concentration of imipramine is quite low. In the current study, it was of interest to us to develop a reliable HPLC method using UV detection and LLE for determination of imipramine in human plasma in the range of 3-40 ng/ml. The described method does not utilize fluorescent detection and yet more sensitive, making the method rapid, simple and appropriate for pharmacokinetic and bioequivalence studies of this drug. This method was validated for its accuracy, precision, limit of detection (LOD), LOQ, robustness and stability as per ICH guidelines.

## MATERIALS AND METHODS

### *Chemicals and reagents*

Imipramine, trimipramine, clomipramine, verapamil, and nortriptiline were purchased from Sigma Chemical Co. (St. Louis, MO). Methanol, hexane, isoamyl alcohol and acetonitrile were purchased from Caledon (Ontario, Canada). All reagents and solutions were either HPLC or analytical grades. Imipramine 25 mg tablets (Batch No:555) manufactured by Lorestan pharmaceutical company (Iran), and Tofranil®25 mg tablets (Batch No:8006) was from Novartis (Switzerland).

### *Chromatographic conditions*

The apparatus used was a Waters HPLC system model 746 (USA), consisting of a model 515 intelligent solvent delivery pump, a 50 µl injection loop, a computerized system controller, and a Waters 2487 UV detector. Chromatographic separation was performed using a µ-Bondapak $C_{18}$ column (250 × 4.6 mm, Waters, Ireland) heated at 40 °C. The mobile phase consisted of sodium hydrogen phosphate solution (0.01 M)/acetonitrile (60/40 v/v) at pH 3.5 ± 0.1. The mobile phase was eluted at a flow rate of 1.5 ml/min and effluent was monitored at 252 nm. Quantitation was achieved by measurement of the peak area ratios of analyte to the internal standard.

### *Choice of internal standard*

To achieve a suitable internal standard for plasma analysis, four drug substances including clomipramine, verapamil, nortriptiline, and trimipramine were examined.

### *Standard solutions of imipramine and internal standard*

Accurately weighted 11.36 mg imipramine hydrochloride (equal to 10 mg imipramine) was added to a 100 ml volumetric flask and dissolved in methanol to obtain a standard stock solution with concentration of 100 µg/ml of pure imipramine.

For construction of the calibration curve, a series of working solutions at concentrations of 60, 100, 200, 400, 600, and 800 ng/ml were prepared by further dilution of the standard stock solution in deionized water. Internal standard stock solution of 3000 ng/ml trimipramine was also prepared in deionized water.

***Calibration curve***

For construction of calibration curve, 100 µl of imipramine working solutions at concentrations of 60-800 ng/ml and 100 µl of trimipramine, as the internal standard, at fixed concentration of 3000 ng/ml were added to test tubes containing 2 ml of human blank plasma to obtain imipramine standard concentrations ranging from 3-40 ng/ml. 200 µl of sodium hydroxide 10 N and 4 ml mixture of hexane/isoamyl alcohol (98:2) were added to each tube, vortexed and centrifuged at 3000 rpm for 10 min. The upper organic layer was transferred to the clean test tubes. The extraction procedure was repeated with another 4 ml mixture of hexane/isoamyl alcohol and added to the tubes and evaporated to 1 ml under nitrogen gas. Then, 100 µl of 0.25 M hydrochloric acid was added to the test tubes. The solution was shaken for 3 min and centrifuged at 7000 rpm for 5 min. The upper layer was discarded and 50 µl of the acid layer was injected into the HPLC system. The calibration curve was constructed by plotting peak area ratios of the drug to the internal standard versus different imipramine plasma concentrations.

***Method validation***

*Linearity*

Calibration plots were constructed using five different concentrations of each sample on five separate days. Analyte to internal standard peak area ratios were plotted against the corresponding concentrations. Distribution of the residuals (percent of difference of the back-calculated concentration from the nominal concentration) was determined to validate the correlation. The calibration model would be accepted if the residuals are within ± 20% for lower limit of quantification and within 15% for all other calibration levels and at least 2/3 of the standards meet this criterion (20). The calibration curves were evaluated by correlation coefficient, slope, and intercept.

*Precision, accuracy, and recovery*

The intra- and inter-day variations of the assay were determined by replicate analysis (n = 5) of samples at concentrations within the range of calibration curves in a single analytical run on the same day and at five different days, respectively, using the same stock solutions and plasma batches. Percentage coefficient of variation (CV%) was used as the measure of precision, and percentage accuracy was also determined. The recoveries of imipramine at the concentration range of calibration curve were evaluated by comparison of the peak areas obtained after extraction of known amount of imipramine from plasma with those obtained from the same amounts of unextracted drug in mobile phase.

***Limit of detection and limit of quantitation***

The parameter LOD was determined using the signal-to-noise ratio by comparing results of the test of samples with known concentrations of analyte to blank samples. The analyte concentration that produced a signal to noise ratio of 3:1 was accepted as the LOD. The LOQ was identified as the lowest plasma of the standard curve that could be quantified with acceptable accuracy, precision, and variability.

***Robustness***

The robustness of the HPLC method was determined by analysis of samples under a variety of conditions such as small changes in the percentage of mobile phase acetonitrile, in the pH, in the mobile phase flow rate, and temperature. The effect on retention time and peak parameters were studied (21).

***Stability***

Plasma samples containing imipramine at three levels of concentration, 0.025, 1, and 4 µg/ml were subjected to short-term (12 h) incubation at room temperature, three freeze/thaw cycles, and storage for 3 months (-20 ºC) (22).

***Application of the method***

The present method was applied in a randomized crossover bioequivalence study in which the concentration of imipramine was measured in plasma samples from twenty healthy adult male volunteers after single oral doses (2 × 25 mg) of two different imipramine tablets from either Tofranil® (reference, Novartis, Switzerland) or imipramine (test, Lorestan, Iran) pharmaceutical companies under fasting conditions. After a two-week wash-out period, the subjects were crossed-over. The study was conducted in accordance with ethical principles and standards described in the Declaration of Helsinki and the International Conference on Harmonization (ICH)/Good Clinical Practice (GCP). Guidelines were approved by an independent Medical Bioethics Committee at the Isfahan University of Medical Sciences. Blood samples (3 ml) were drawn from the forearm at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 24, 36, 48 and 60 h after administration, transferred to heparinized tubes, and gently mixed. After centrifugation, the separated plasma samples were collected and stored at -20 ºC prior to analysis.

***Pharmacokinetic analysis***

The peak plasma concentration ($C_{max}$) and the corresponding peak time ($T_{max}$) were obtained directly from individual plasma concentration–time profiles. The area under the curve ($AUC_{0–60}$) was calculated by the trapezoidal rule and the total $AUC_{0–\infty}$ was calculated according to the following equation:

$$AUC_{0-\infty} = AUC_{0-60} + C_{60} / K_E \quad (1)$$

where, $C_{60}$ is the drug concentration after 60 h and $K_E$ is the elimination rate constant.

***Statistical analysis***

For the purpose of bioequivalence analysis, $AUC_{0–60}$, $AUC_{0–\infty}$, $C_{max}$, and $T_{max}$ were considered as primary variables. For each of the parameters, the values obtained for the two products were subjected to analysis of variance (ANOVA) to assess the effect of treatment (formulation), periods, sequences, and subjects on the parameters.

The 90% confidence interval should generally be within the acceptance range 80% to 120%. Non-parametric Wilcoxon signed rank test for paired samples was used to compare values of $T_{max}$ of the test over the reference products. All statistical analyses were performed using SPSS 17.

## RESULTS

***Calibration curve***

Acceptable linear relationships was found in the range of 3-40 ng/ml concentrations. Since distribution of residuals was within ± 5%, no weight factor was applied. Although blank plasma samples (with a zero concentrations) were not selected in constructing the calibration curves, the 95% confidence interval of the intercept encompassed the origin. The linear regression equation was Y = 0.0133X (± 0.001) + 0.0079 (± 0.0004) with the mean correlation coefficient ($r^2$) of 0.999 ± 0.0022.

***Limit of detection and limit of quantitation***

The LOD and LOQ were found to be 1 ng/ml and 3 ng/ml, respectively. Representative chromatograms of blank human plasma spiked with internal standard (a), the lowest standard concentration and internal standard (3 ng/ml) (b), and human plasma sample 6 h after oral ingestion (c) of 50 mg imipramine are shown in Fig. 1. No interfering substances were observed at the retention time of imipramine and internal standard and both compounds were eluted completely and appeared as two separate resolved peaks (Resolution factor: 1.36) without peak tailing (tailing factor: 1.03, 1.22, respectively). An optimum flow rate of 1.5 ml/min for the mobile phase resulted in the retention times of about 4.3 min for imipramine and 5.1 min for internal standard. Clomipramine as one of the potential internal standard was eluted at 5.5 min but its low extraction recovery (less than 30%) contributes to the nonreproducible results. Though verapamil was eluted before imipramine but interfered with one of the imipramine metabolite. The analytical peak of nortriptyline appeared in the same region as the peak of imipramine. Amongst potential internal standards examined, trimipramine met all the typical requirements of a compound to be used as an internal standard.

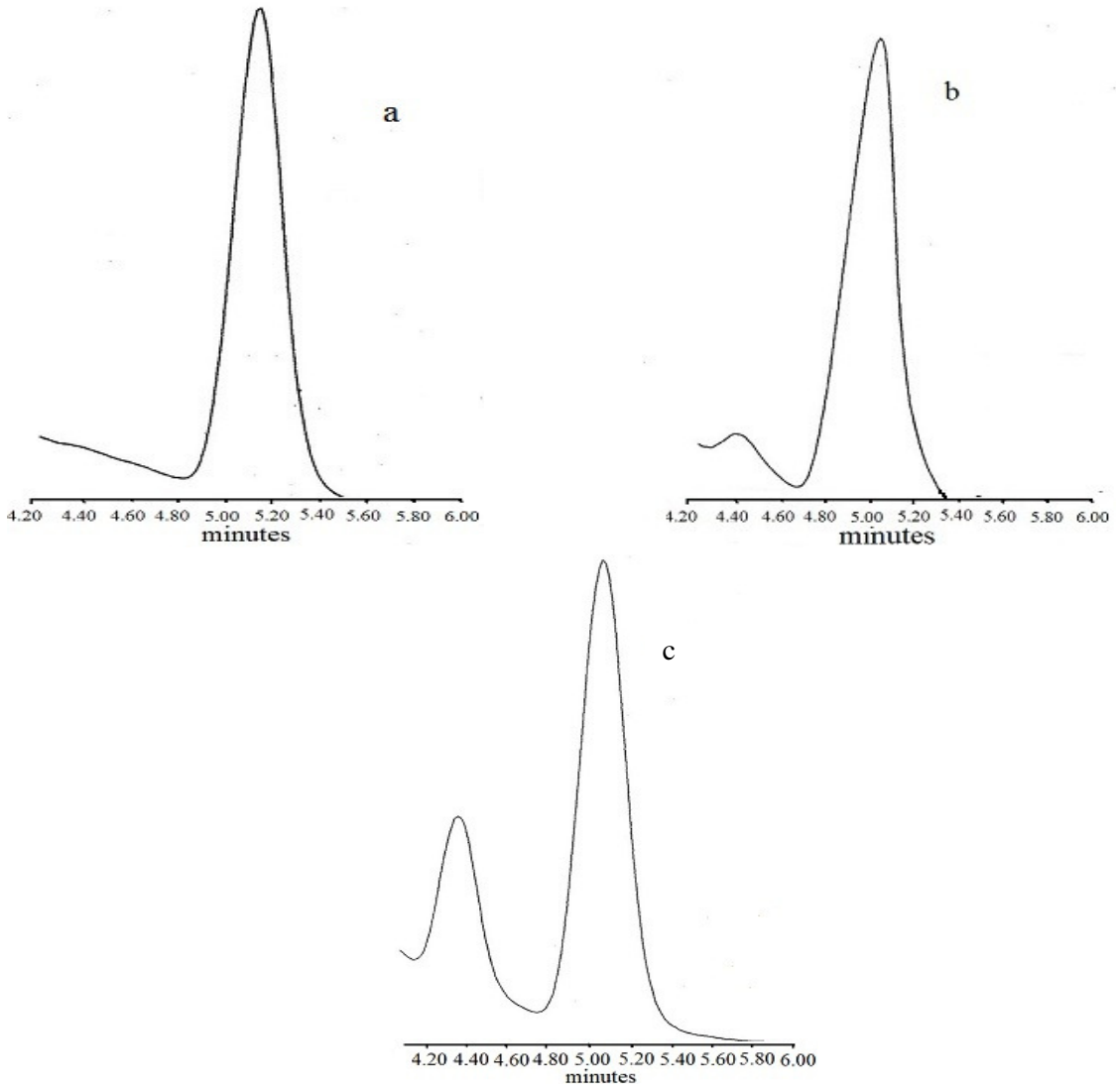


**Fig. 1.** Representative chromatograms of a; blank control plasma containing internal standard at a nominal concentration of 150 ng/ml, b; plasma containing 3 ng/ml imipramine and 150 ng/ml internal standard, and c; human plasma 6 h after oral ingestion of 50 mg imipramine.

This agent is stable, readily available, adequately extracted from plasma (around 70%), well resolved from imipramine (resolution factor: 1.21), and its peak was sharp and symmetric (tailing factor: 1.22).

***Precision, accuracy and recovery***

The precision and accuracy of calibration standard concentrations were within acceptable limits as defined in the ICH guidelines. The inter- and intra-day precision and accuracy values of the assay method are presented in Table 1. The mean recoveries (accuracy) of imipramine after plasma extraction were 97.4% ± 3.6.

***Robustness***

The results of the robustness of the assay method are listed in Table 2. The tailing factor for imipramine and trimpramine always ranged from 1 to 1.3 and the eluents were well separated under all the changes carried out (resolution factor: 1.12-1.46). The percent recoveries of imipramine were good under most conditions and did not show a significant change when the critical parameters were modified. Considering the result of modifications in the system suitability parameters and the specificity of the method, it would be concluded that the method conditions are robust.

**Table 1.** The intra- and inter-day variability of the HPLC assay for determination of imipramine concentration in human plasma.

| Nominal Concentrations (ng/ml) | Inter-day variability | | | | Intra-day[c] variability | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean | SD[a] | CV[b] (%) | Accuracy (%) | Mean | SD | CV (%) | Accuracy (%) |
| 3 | 2.77 | 0.30 | 10.8 | 92.3 | 3.10 | 0.47 | 15.4 | 103 |
| 5 | 4.95 | 0.25 | 5.09 | 99.1 | 5.11 | 0.58 | 11.6 | 102 |
| 10 | 9.56 | 0.42 | 4.43 | 95.6 | 10.1 | 0.87 | 8.61 | 101 |
| 20 | 20.1 | 0.30 | 1.52 | 100 | 20.6 | 1.35 | 6.56 | 103 |
| 30 | 29.6 | 1.08 | 2.72 | 98.6 | 28.8 | 2.24 | 7.43 | 96 |
| 40 | 40.8 | 0.83 | 2.02 | 102 | 40.4 | 2.33 | 5.78 | 101 |

[a]Standard deviation, [b]Coefficient of variation, [c]Data are presented only for the 3rd day of the experiment.

**Table 2.** Influence of changes in experimental parameters on the performance of chromatographic system.

| Parameter | Modification | Recovery* | Tailing factor | Resolution factor |
|---|---|---|---|---|
| Mobile phase ratio (v/v) Buffer:Acetonitrile | 65:35 | 85.6 | 1.08 | 1.14 |
| | 63.5:37.5 | 99.1 | 1.05 | 1.28 |
| | 60:40 | 87.4 | 1.03 | 1.36 |
| | 57.5:42.5 | 89.5 | 1.12 | 1.28 |
| | 55:45 | 89.1 | 1.29 | 1.26 |
| PH | 4 | 89.8 | 1.06 | 1.24 |
| | 3.5 | 87.4 | 1.03 | 1.36 |
| | 3 | 85.5 | 1.12 | 1.21 |
| Flow rate (ml/min) | 1.8 | 88.9 | 1.05 | 1.13 |
| | 1.5 | 87.4 | 1.03 | 1.36 |
| | 1.2 | 82.2 | 1.23 | 1.22 |
| Temperature ($^{o}$C) | 50 | 88.8 | 1.05 | 1.28 |
| | 40 | 87.4 | 1.03 | 1.36 |
| | 30 | 91.7 | 1.15 | 1.23 |

*Recovery = (measured concentration/nominal concentration) ×100

**Table 3.** Comparison between pharmacokinetic parameters of two products of imipramine in healthy human volunteers.

| Pharmacokinetic parameters | *$C_{max}$ (ng/ml) | *$AUC_{0-60}$ (ng/ml).h | *$AUC_{0-\infty}$ (ng/ml).h | $T_{max}$ (h) |
|---|---|---|---|---|
| Test (Mean ± SD) | 27.63 ± 1.79 | 549.4 ± 79.16 | 712.9 ± 126 | 3.17 ± 0.75 |
| Reference (Mean ± SD) | 28.35 ± 2.32 | 561.3 ± 63.69 | 710.1 ± 84.1 | 3.08 ± 0.85 |
| *CI 90% | 93.2 - 102.7 | 90.6 – 108.5 | 90.2 – 113.8 | $P$>0.05 |

*Parameters were not logarithmically transformed to calculate the confidence intervals.

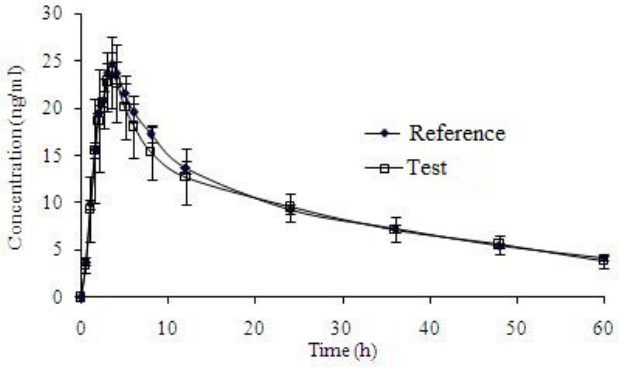


**Fig. 2.** Plasma imipramine profiles following oral administration of two different imipramine tablets to healthy human volunteers.

***Stability***

Treated plasma samples were found to be stable at least 24 h when the samples were kept at room temperature (error percent <5.4%). The concentrations of imipramine in plasma which underwent three freeze-thaw cycles or storage at -20 ºC for 30 days were found to be stable with relative error of less than 8.2%.

***Bioequivalence study in healthy human volunteers***

The concentration–time profiles following oral administration of two different imipramine tablet products are plotted in Fig. 2. The mean pharmacokinetic parameters for the brands of imipramine tablets are summarized in Table 3. The pharmacokinetic parameters obtained in this study are in accordance with those reported by some other researchers (5,14,18). The mean $AUC_{0–60}$, $AUC_{0–\infty}$, and $C_{max}$ of the test product (imipramine) were compared with those of reference (Tofranil®). ANOVA did not reveal any significant differences in periods, formulations, or sequences (all *P* values >0.05). As shown in Table 3, 90% confidence intervals for nontransformed values of $AUC_{0–60}$, $AUC_{0–\infty}$, and $C_{max}$ of the test over those of the reference product were found to be within the FDA acceptable range of 0.8–1.20 for bioequivalence evaluation.

## DISCUSSION

To characterize pharmacokinetics and bioequivalence studies of imipramine in human plasma, a sensitive, specific and reproducible HPLC method was developed and validated in our laboratory. A literature survey indicated that studies on HPLC measurement of imipramine in biological samples following LLE with UV detector are scarce and the quantification limits have not been sufficiently low to precisely characterize the drug plasma profiles (11,13,14).

The inherent fluorescence of imipramine allowed the use of sensitive fluorescence detector with HPLC to measure plasma levels of this drug (5,12). Although, the methods described in these reports are sufficiently sensitive and accurate for the measurement of imipramine and indicated short chromatographic run time, they require extremely pure solvents and are not readily available and affordable at most laboratories. The very low quantification limit of 3 ng/ml obtained in the current study is less than that of most previous reports even when fluorescence detection was used which allowed us to avoid using fluorometric detection. UV detection produces more reproducible responses in comparison with fluorescence detectors. Another advantage of the method developed in the present study is efficient LLE of the drug from plasma which renders the method more convenient and faster for pharmacokinetic and bioequivalence studies of imipramine. Although mild acidic solution was finally injected in to the injection port, it did not seem to cause any problem to the column or injection port. The method described in this report has been used for two years in a research setting. Several hundred injections have been made on a single column with no effect on column durability or HPLC injection port. The use of guard column greatly extends the analytical column life. Among different extracting solvents examined, only the mixture of hexane/isoamyl alcohol (98:2 *v/v*) resulted in excellent recoveries around 85% for the drug. In order to achieve highest resolution under isocratic condition, the mixtures of methanol and/or acetonitrile with sodium hydrogen phosphate solution with various combinations were assessed as mobile phase. Binary mixture of phosphate solution/acetonitrile in proportion of 60/40 (*v/v*) proved to be the most effective combination as evidenced by more efficient resolution devoid of any tailing. In some previous works (23,24) the gradient mode was used to resolve imipramine peak from internal standard peak, in which composition of mobile phase should be changed throughout the run. However, gradient method is more complex technique and is not sufficiently reproducible compared to isocratic method. The HPLC assay developed in the current study was successfully used in pharmacokinetics

and bioequivalence studies of imipramine tablets**.** Based on estimated pharmacokinetic parameters and statistical analyses, it was found that imipramine tablets manufactured by Lorestan company is bioequivalent to Tofranil®, manufactured by Novartis company, and that both products can be considered equally effective in medical practice. The estimated pharmacokinetic parameters found to be in accordance with previous reports.

## CONCLUSION

The present investigation describes a simple, sensitive and selective HPLC method for analyses of imipramine in plasma samples with LLE and UV detection. The mixture of hexane/isoamyl alcohol as extracting solvent gave excellent recovery for the drug. The method was validated and met the requirements of linearity, recovery, accuracy, precision, and robustness. This analytical procedure could readily be applied to routine analysis of plasma samples for bioavailability and pharmacokinetic studies of imipramine in human.

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