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# Fast urinary screening for imipramine and desipramine using on-line solid-phase extraction and selective derivatization

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**Abstract**

A continuous-flow configuration based on sequential solid-phase extraction and derivatization is proposed for the screening of urine samples for imipramine and related metabolites. For the first time, a 50/50 (v/v) methanol/nitric acid mixture is used as both eluent and derivatizing reagent. Sample aliquots are injected into the flow manifold and driven by a water stream to an RP-$C_{18}$ column where the drugs are quantitatively retained. Following clean-up step with 40/60 (v/v) methanol/water, the eluent/derivatizing reagent is injected and passed through the sorbent column, eluted drugs reacting with nitric acid to form a blue dye that is monitored at 600 nm. The global signal thus obtained for the antidepressants can be used to estimate their total concentration in the samples without the need to individually quantify the analytes. This total index can be used for timely decision-making in case of overdosage. The proposed method is sensitive and selective; thus, typical interferents such as endogenous and diet compounds have no substantial effect on the analytical signal. This allows imipramine and its metabolites to be determined at therapeutic levels in urine samples.


*Keywords:* Imipramine; Desipramine; Tricyclic antidepressants; Urine samples; Flow analyser; Solid-phase extraction; Derivatization

## 1. Introduction

Tricyclic antidepressants (TCAs) are widely used for the treatment of various psychiatric disorders including neuropathic pain [1] and also to prevent enuresis in children [2]. In therapeutic doses, TCAs block post-synaptic receptors and inhibit re-uptake of various neurotransmitters such as histamine, dopamine, acetylcholine, serotonin and norepinephrine. In toxic doses, however, they can cause hypotension and even cardiovascular disease [3].

Chemically, TCAs are secondary or tertiary amines with a common core consisting of two aromatic rings fused with a seven-atom ring which may or may not include a nitrogen heteroatom (see Fig. 1). Tertiary amines are readily demethylated *in vivo* to the corresponding secondary amines, which can be also active. The metabolic pathway for the most widely used TCA, imipramine, involves the formation of several metabolites (*viz.* desipramine, 2-hydroxyimipramine, 2-hydroxydesipramine and didesmethylimipramine), which are released mainly through urine [4].

Liquid chromatography [5–10] or capillary electrophoresis, in combination with UV–vis detection, constitutes the usual choice for analysing bioanalytical samples [11–14]. Gas chromatography with mass spectrometric detection has been used to determine imipramine and desipramine in human plasma [15]. Solid-phase extraction (SPE) [5,13], solid-phase microextraction (SPME) [6,9], fiber-in-tube-SPME [7,8,13], supported liquid membrane extraction [10] and dialysis [11] have been proposed for sample processing with a view to ensuring the sensitivity and selectivity levels required for the individual determination of TCAs. The previous chromatographic and electrophoretic methods have provided quantitation limits ranging from 0.05 to 1 $\mu g\,mL^{-1}$ for a variety of TCAs (imipramine, desipramine, amitriptyline and nortriptyline, mainly). The need for extensive sample treatment strongly reduces the throughput of the overall process, which can have an adverse impact on timely delivery of results by clinical laboratories.

Chemometrics has proved a powerful tool for resolving mixtures of chemical compounds by using a multi-dimensional instrumental technique such as UV–vis spectroscopy [16,17].

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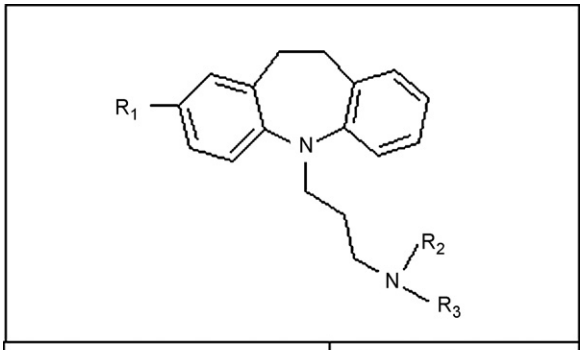


| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| Imipramine | H | $CH_3$ | $CH_3$ |
| Desipramine | H | H | $CH_3$ |
| 2-hydroxyimipramine | OH | $CH_3$ | $CH_3$ |
| 2-hydroxydesipramine | OH | H | $CH_3$ |
| Didesmethylimipramine | H | H | H |

Fig. 1. Chemical structures of imipramine and related metabolites.

However, chemometric methods are poorly selective and usually applicable to a small number of analytes only.

The redox properties of TCAs have traditionally been used for their determination, even though the ensuing methods are not selective enough to provide a response which can be unambiguously assigned to the drugs present in the sample. This has severely restricted the scope of these methods for quality control in the pharmaceutical industry, where matrices are simpler and better defined than in the clinical domain. For example, TCAs can be reacted with Fe (III) and 2,2′-bipiridine to give a pink colour the formation of which can be monitored at 530 nm [18]. An indirect fluorimetric method based on the oxidation of these drugs with Ce(IV) in acid medium and measurement of the fluorescence of the resulting Ce(III) ion is also available [19]. Finally, TCAs can be determined conductimetrically by formation of ion-associated complexes [20].

In this work, we developed a new method for the determination of imipramine and its main metabolites in urine samples. The method uses a flow configuration to preconcentrate the analytes on octadecyl silica sorbent for their subsequent elution and derivatization. The reaction between the analytes and nitric acid present in the eluent yields a blue dye that can be monitored photometrically. The determination is quite fast (*ca.* 12–15 min), and acceptably sensitive and selective; also, it requires no sample treatment other than pH adjustment and filtration. This makes the proposed method much faster than its chromatographic and electrophoretic counterparts.

## 2. Experimental

### 2.1. Reagents and samples

All reagents used were analytical-grade or better. The tricyclic antidepressants (imipramine and desipramine) were supplied by Sigma–Aldrich (Madrid, Spain). HPLC gradient grade methanol (Panreac, Barcelona, Spain), 65% nitric acid (Scharlau, Barcelona, Spain) and Milli-Q ultrapure water (Millipore Corp, Madrid, Spain) were also employed. The eluent/derivatizing reagent (*viz.* 50/50 (v/v) methanol/nitric acid) and washing solution (40/60 (v/v) methanol/water) were both prepared on a daily basis by carefully mixing the appropriate solvents. All solutions were sonicated in an ultrasound bath prior to use. Phosphate buffered saline tablets and sodium hydroxide, purchased from Sigma–Aldrich (Madrid, Spain), were employed to adjust the pH of the aqueous solutions and samples to a final value of 8.1.

Individual stock standards of the TCAs at a final concentration of 5 mg $mL^{-1}$ were made by dissolving appropriate amounts of the chemicals in Milli-Q water. Working-strength solutions were prepared on a daily basis by careful dilution of the stock solutions in phosphate buffer.

Blank urine samples were collected from healthy individuals and stored in appropriate polyethylene flasks at −20 °C until analysis. Each sample was pH-adjusted and passed through a disposable syringe nylon filter of 0.45 μm pore size from Millipore (Madrid, Spain) prior to injection into the flow system.

### 2.2. Apparatus

The automated configuration used in this work (Fig. 2) was a typical flow injection manifold on-line connected to a spectrophotometer. A Hewlett-Packard 1100 high-pressure binary gradient pump (Palo Alto, CA) was used to deliver the phosphate buffer and 40/60 (v/v) methanol/water streams. Two six-port low-pressure injection valves (Rheodyne, Cotati, CA) fitted with loops of 5 and 1 mL were employed for samples and the eluent/derivatizing reagent, respectively. The derivatization products were monitored with a Hewlett-Packard HP 1100A UV–vis detector, using a fixed wavelength of 600 nm. Signals were acquired and processed by using HPChem Station software (Agilent Technologies) and peak areas as the analytical signals.

The flow manifold was constructed from PTFE tubing of 0.5 mm i.d. for coils in addition to standard connectors. A laboratory-made solid-phase extraction column (2.5 cm × 3 mm i.d.) packed with *ca.* 40 mg of octadecyl silica sorbent (Bond Elut, Varian, Madrid, Spain) and fitted with small cotton beads at both ends to prevent material losses was used to preconcentrate the analytes. Finally, a Selecta 50 W, 60 Hz ultrasonic bath was used to degas solvents.

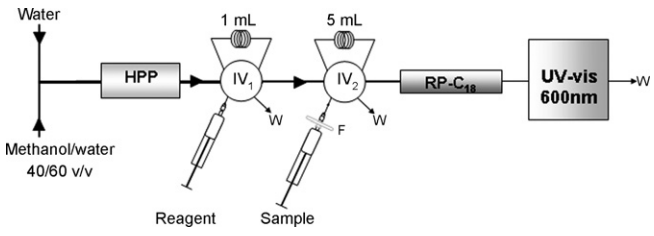


Fig. 2. Schematic diagram of the continuous-flow configuration used for the determination of tricyclic antidepressants in urine samples. HPP, high-pressure pump; IV, injection valve; F, on-line nylon filter (0.45 μm); RP-$C_{18}$, octadecyl silica sorbent column; UV–vis, photometric detector; W, waste.

### 2.3. Analyser operation

The analyser used to quantify imipramine and its metabolites in urine samples was operated in a sequential fashion. Thus, urine samples were passed through a commercial syringe filter (nylon, 0.45 μm pore size). Initially, the loops of the injection valves were filled with eluent/derivatizing reagent (*viz.* 50/50 (v/v) methanol/nitric acid, $IV_1$) and aqueous standards/urine samples ($IV_2$) by means of plastic syringes. Then, by switching $IV_2$, the sample was injected into an aqueous phosphate buffer solution and driven to the RP-$C_{18}$ column at a flow rate of 1.0 mL $min^{-1}$ for analyte retention (5 min). A clean-up operation was needed to remove potential interferents remaining on the sorbent column. For this purpose, a 40/60 (v/v) methanol/water stream was passed through the system at a flow rate of 1.4 mL $min^{-1}$ for 4 min. Finally, switching $IV_1$ caused the eluent/derivatizing reagent to be injected and driven to the sorbent column at a flow rate of 1 mL $min^{-1}$ for the sequential elution and derivatization of the TCAs. The blue dyes thus formed were detected photometrically.

The determination of the analytes was completed within 10 min. After each analysis, a fresh phosphate buffer solution stream was passed through the system at a flow rate of 1 mL $min^{-1}$ (5 min) to remove any excess methanol/nitric acid mixture and make the system ready for a new injection. Under these conditions, the column remained useful for at least 10 working days.

## 3. Results and discussion

### 3.1. Selection of the determination method

Tricyclic antidepressants undergo irreversible oxidation in nitric acid at room temperature [21] and the resulting blue dye can be used to determine the drugs. The sole structural requirement for this reaction is the presence of a nitrogen atom in the central ring, which seems to play a key role in the process. The reaction is useful with both imipramine and its main urinary metabolites (Fig. 1); this allows a global index (imipramine + related metabolites) to be determined for biological samples.

Although the reaction is very fast, the product is unstable and decomposes very rapidly. Therefore, ensuring reproducibility in its determination entails using a fixed measurement time. The proposed flow configuration can be quite effective for this purpose as measurements are made at a fixed time for improved precision and shortly after derivatization in order to maximize the analyte response before the reaction product can degrade to a significant extent.

A solid-phase column was included in the system in order to improve the sensitivity and selectivity by effect of interferents being removed and analytes preconcentrated.

As in batch work, implementing the derivatizing reaction in the continuous-flow systems was expected to require the use of Fe(II) – which would be oxidized to Fe(III) in the nitric medium – as a catalyst for the reaction between TCAs and nitric acid. However, the addition of a minimal concentration of Fe(II) (30 mg $L^{-1}$) to the eluent/derivatizing reagent was found to enable the formation of the blue dye in the flow system.

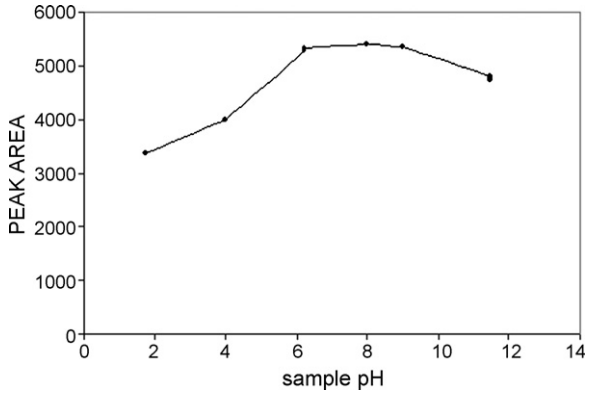


Fig. 3. Influence of sample pH on retention of the studied TCAs on the $C_{18}$ column.

### 3.2. Optimization of experimental variables

The proposed flow configuration (Fig. 2) was optimized by using a working standard solution containing imipramine at a final concentration of 1 μg $mL^{-1}$. The influence of chemical (sample pH, sorbent material, eluent/derivatizating reagent), hydrodynamic (flow rates and amount of sorbent) and instrumental variables (wavelength) was examined in depth.

A preconcentration step was required prior to analytical measurements owing to the low concentration of the target analytes in urine samples. The best choice in this respect involved on-line coupling SPE with detection. Based on the chemical structures of TCAs, we tested various commercially available sorbents including Octadecyl silica, StrataX and Lichrolut EN. The influence of the sample pH was studied over the range 2–10 by adding appropriate amounts of hydrochloric acid or sodium hydroxide to the aqueous standard solutions (Fig. 3 illustrates the influence of this variable on the analytical signal).

Analyte retention and elution were carefully studied, with special emphasis on the possibility of performing elution and derivatization simultaneously. The elution/derivatizing solution was prepared in various solvents of which methanol/nitric acid mixtures were found to provide the best results. The proportion of each mixture component proved a critical variable as the mixture should contain enough methanol to elute all analytes and enough nitric acid to complete the derivatization reaction. The best results were obtained by using a sample pH of 8.1 with an octadecyl silica column for retention and a 50/50 (v/v) methanol/nitric acid mixture as the eluent/derivatizing reagent.

The sensitivity of the determination was thought to be affected by two hydrodynamic variables, namely: the sample and reagent flow rates. Both variables were studied over the range 0.2–2.0 mL $min^{-1}$ and a value of 1.0 and 1.4 mL $min^{-1}$ found to be the optimum choice for the sample and reagent, respectively (see Fig. 4). As regards the amount of sorbent, 40 mg was found to suffice in order to ensure quantitative retention and elution of the drugs at therapeutic levels in urine samples. Silica-

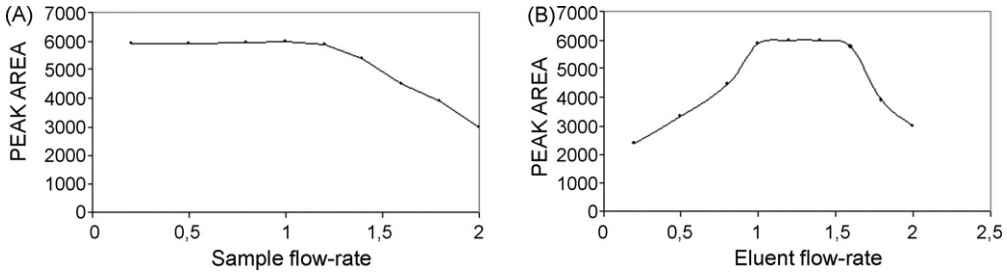


Fig. 4. Effects of the flow rate of sample (A) and eluent-derivatizing reagent (B) on the analytical signal for the TCAs.

based sorbents are known to be unstable in extremely acid and alkaline media. Thus, very acidic media break down bonds in silica stationary phases. Because the derivatizing reagent consisted of nitric acid, this required studying the stability of the sorbent column. To this end, a standard solution containing a 1 μg mL$^{-1}$ concentration of imipramine was injected into the system three times on ten consecutive days ($n = 30$). Based on the results, the material remained stable for at least 10 days. Thus, the variation of the analytical signal (peak area) was less than 6% and the average within-day precision better than 3.3%; also, the retention time for the monitored peak ranged from 10.7 to 10.8 min between days, with a within-day precision better than 1%. Based on these results, the column was replaced on a weekly basis in order to ensure adequate accuracy results.

The absorption spectrum for the blue dye formed in the analytical reaction absorbed maximally at *ca.* 630 nm; however, we chose to use 600 nm as the monitored wavelength since this was the maximum value afforded by the detector.

### 3.3. Analytical performance of the proposed configuration

The absorptiometric behaviour of the target analytes under flow conditions is a critical aspect in the proposed methodology. This led us to examine it in depth before constructing any calibration graphs. The behaviour of the analytes should be as similar as possible in order to obtain an accurate global response directly related to the analyte concentrations from a single calibration graph. A simple test was used for this purpose based on imipramine and its main metabolite, desipramine, as model analytes. Two series of standard solutions containing only one of the analytes at variable concentrations were analysed with the analyser for subsequent comparison. The results revealed the absorptiometric behaviour of the analytes to be quite similar judging by the slope of the curve obtained from molar concentrations (1.05). This conclusion is rather important as it demonstrates that the reaction between the analyte and nitric acid is not strongly dependent of the chemical structure of the analytes and also that the analytical signal is not affected by slight differences. This allows the determination of a global index for imipramine and related metabolites in the sample provided only one analytical signal is obtained. Moreover, desipramine itself is a metabolite of other TCAs such as lofepramine, which should allow one to detect the presence of TCAs via their metabolic product and extend the scope of the proposed method as a result.

Based on the foregoing, we chose to use desipramine as model compound to assess the analytical figures of merit of the proposed method (Table 1). The calibration graph was constructed by injecting standard solutions containing variable concentrations of the drug over the range 0.05–2.00 mg L$^{-1}$ into the analyser (Fig. 2), using three replicates per standard at eight concentration levels each. Calculating detection and quantitation limits from a non-linear signal–concentration response is difficult. In order to simplify the calculations, we fitted a linear relationship to the lower portion of the calibration curve (0.05–0.30 mg L$^{-1}$) and used both the standard deviation of the estimate ($S_{y/x}$) and intercept ($S_a$). The limits of detection (LD) and quantification (LQ) were calculated as three and ten times, respectively, the standard deviation divided by the slope of the graph. The results were similar with both $S_{y/x}$ and $S_a$. Also, using the criterion adopted by the US Environmental Protection Agency (US EPA) [22], which revolves around a parameter called the "method detection limit" (MDL), led to a similar value for the lowest detectable concentration (18.2 ng mL$^{-1}$).

The precision (repeatability) of the method, expressed as relative standard deviation, was assessed by injecting 11 replicates of a standard solution containing desipramine at a 0.20 mg L$^{-1}$ concentration.

Table 1
Figures of merit of the proposed analyzer for the determination of desipramine in real urine samples

| | | | |
|---|---|---|---|
| Regression equation[a] | $y = (4572.5 \pm 131.7)C^2 + (2418.8 \pm 61.7)C + (5.0 \pm 4.1)$ | | |
| Desipramine | | | |
| $S_{y/x} = 105.4$ | $R = 0.9999$ | Dynamic calibration range[b] = 0.05–2.00 | |
| LD[c] = 15 | LQ[c] = 50 | RSD (%) = 3.3 | Concentration levels = 8 (by triplicate) |

[a] y = peak area; $C$ = concentration (mg L$^{-1}$).
[b] mg L$^{-1}$.
[c] μg L$^{-1}$.

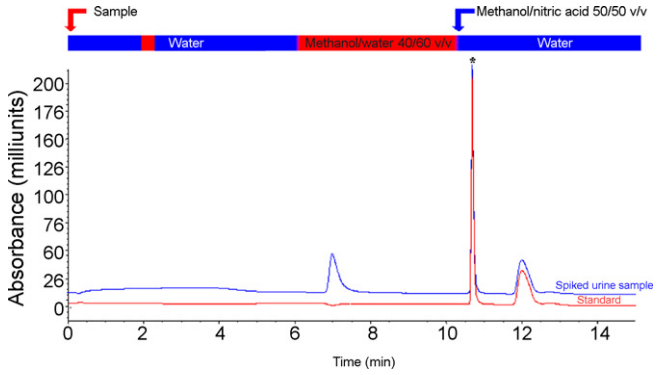


Fig. 5. Typical flow profiles obtained from a spiked urine sample and a standard solution containing imipramine at identical concentrations. The analyte peak is marked with an asterisk.

### 3.4. Analysis of urine samples

The oxidation reaction is non-specific for TCAs; in fact, other reducing compounds present in urine such as endogenous components and other drugs can interfere with it by competing with the reagent or resulting in spectral overlap. In addition, urine is highly coloured (especially under acidic conditions), so it can interfere with absorptiometric measurements. Some interference can therefore be encountered if the previous compounds are retained on the sorbent column and subsequently eluted from it. Cleaning up the sorbent material in the SPE procedure is therefore crucial with a view to increasing the selectivity by removing as many interferences as possible without eluting the target analytes. We used various solvent mixtures and found 40/60 (v/v) methanol/water adjusted to pH 8.1 to provide the best results. Fig. 5 shows a typical flow profile for a spiked urine sample and a standard solution both containing imipramine at the same concentration level. The upper part of the figure shows the gradient of solvents delivered by the high-pressure pump together with the injection times for the sample (or standard solution) and elution/derivatizing reagent. Injecting a spiked urine sample resulted in an increase (from 0 to 5 min) in absorbance value due to unretained components of the sample matrix. A new peak at retention time of *ca.* 7 min appeared as the washing solution (40/60 (v/v) methanol/water) was passed through the column that corresponded to the matrix compounds retained on the sorbent and eluted by the mixed solvent. Finally, injecting the eluent/derivatizing reagent yielded a peak at a retention time of *ca.* 10.7 min the area of which was proportional to the concentration of the target analyte (imipramine in this case).

Because no urine samples containing the studied TCAs were available, a recovery test was performed on blank urine samples in order to assess the applicability of the proposed method. Variability among samples was ensured by collecting them from three different donors. The accuracy of the method was assessed by using two types of calibration curves. Thus, an ex-matrix calibration curve was constructed from pure desipramine standards at different concentration levels (0.10, 0.20, 0.40, 0.60, 0.80, and 1.00 mg $L^{-1}$) and an in-matrix calibration curve obtained by using three different blank urine samples spiked at the same concentration levels as the standards. All samples were analysed in triplicate ($n = 3$). The regression equation obtained for in-matrix calibration was $y = 3964C^2 + 2885.6C - 33$ ($R^2 = 0.998$). Recoveries were calculated by comparing the results of the two calibration series. Table 2 compares the analyte concentrations added to and found in the real samples. Recoveries were acceptable in all instances. A plot of these results in the form $[\text{analyte}]_{\text{found}}$ versus $[\text{analyte}]_{\text{added}}$ was found to be highly linear ($y = 0.978x + 0.016$, $R = 0.9994$). The slope of the linear model corresponds to the average recovery, which was 97.8%. The good fitting obtained also indicates that recovery was quite constant throughout the studied concentration range. A paired *t*-test was also performed on the samples stated in the table; the experimental and critical *t*-value were 0.13 and 2.57, respectively ($P = 0.05$). No systematic differences were thus found, which confirms the good performance of the proposed method

Table 2
Analysis of spiked urine samples using the proposed method

| Sample | Added (μg $mL^{-1}$) | Found (μg $mL^{-1}$) | Recovery (%) | RSD (%) |
|---|---|---|---|---|
| Urine 1 | 0.100 | 0.096 ± 0.004 | 96 ± 4 | 4.2 |
| Urine 2 | 0.20 | 0.21 ± 0.01 | 105 ± 4 | 4.8 |
| Urine 3 | 0.40 | 0.41 ± 0.01 | 101 ± 3 | 2.4 |
| Urine 4 | 0.60 | 0.59 ± 0.02 | 98 ± 3 | 3.4 |
| Urine 5 | 0.80 | 0.81 ± 0.03 | 102 ± 3 | 3.7 |
| Urine 6 | 1.00 | 0.98 ± 0.03 | 98 ± 3 | 3.1 |

## 4. Conclusions

A comparison of the performance of the proposed method with its chromatographic counterparts reveals that the former is substantially more expeditious by virtue of its integrating analyte isolation, derivatization and detection in a single step. This minimizes the effects of the instability of the blue dye formed and results in increased precision and robustness. The simplicity of the information handled allows the method to be used for fast decision-making in the clinical and forensic fields. Unlike analytical methods based on the formation of a coloured derivative, the proposed method is subject to no interference from dietary components (*e.g.* reducing compounds). The sole weakness of our method seems to be its inability to discriminate between individual analytes present in the sample.

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