ORIGINAL ARTICLE

Stability-indicating HPLC method for the determination of nicardipine in capsules and spiked human plasma. Identification of degradation products using HPLC/MS

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Received 3 August 2012; accepted 25 November 2014

Abstract In this stability-indicating, reversed-phase high-performance liquid chromatographic method for nicardipine (NIC), forced degradation has been employed and the formed degradants were separated on a C18 (150 mm × 3.9 mm, 5 μm) analytical column using a mobile phase consisted of 70% methanol: acetic acid containing 0.01 M triethylamine with pH 4. The flow rate was 1.0 mL/min and the photodiode array detection wavelength was 353 nm. Forced degradation of the drug was carried out under acidic, basic, photolytic, and oxidative stress conditions. Chromatographic peak purity data indicated no co-eluting peaks with the main peaks. This method resulted in the detection of seven degradation products. Among these, two major degradation products from basic hydrolysis, one from oxidation by H2O2 and four from photolytic stress were identified by mass spectral data. A good linear response was achieved over the range of 0.5–40 μg/mL with a limit of detection (LOD) of 0.011 μg/mL and limit of quantification (LOQ) of 0.036 μg/mL.

The suggested method was successfully applied for the analysis of NIC in its commercial capsules, with mean% recovery value of 100.11 ± 2.26%. The method was extended to the in vitro determination on NIC in spiked human plasma samples with mean% recovery of 99.04 ± 5.67%. The suggested method was utilized to investigate the kinetics of photolytic induced degradation.

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1. Introduction

Calcium channel blockers (CCBs) are established in therapies for the treatment of high blood pressure, angina, and certain heart rhythm abnormalities. They lower blood pressure by a direct and selective relaxing effect on the smooth muscle cells, especially those of resistance vessels (Michalewicz and Messerli, 1997). This helps to maintain blood flow to the heart
and reduce the frequency and severity of angina attacks. CCBs are used in monotherapy as well as combined with angiotensin-converting enzyme (ACE) inhibitors or β-blockers (Domenic, 2001). Nicardpine (NIC) is one of the CCBs. NIC is a substituted 1,4 dihydropyridine derivative and chemically designed as [2-benzyl(methyl)amino]ethyl methyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate] Fig. 1. Being a dihydropyridine derivative, NIC is widely used in the treatment of hypertension and angina pectoris (Graham et al., 1985).

It is known that calcium antagonists from the group of 1,4 dihydropyridine derivatives are liable to photochemical decomposition to give degradation products that have no pharmacological activity (Brogden and Mc Tavish, 1995; Zhang et al., 1998; Faust, 1993). The degradation is usually brought about by any of the following methods: oxidation, hydrolysis and photo decay.

Several papers can be cited in the literature demonstrating the occurrence of various decomposition products based on HPLC/MS (Chen et al., 2007; Marinkovic et al., 2003; Barandau et al., 2006), GC/MS (Mielcarek et al., 2000) and spectrophotometric (Ragno et al., 2002; Ragno et al., 2006) methods. Drugs that possess certain labile functional groups are prone to decomposition particularly when formulated in liquid preparations. The assay of these formulations can be carried out by a suitable stability indicating method, e.g. HPLC which is by far the method that is most widely used today as a stability indicating method. Stability-indicating methods are required for the assay of drug formulations where the intact drug and its degrading products coexist together.

Stress testing of the drug was also conducted, according to the ICH Guideline to support the suitability of the method. The system suitability was used to verify if the resolution and injection repeatability of the chromatographic system were adequate (ICH-Harmonised Tripartite Guideline, 2003).

Consequently, a study of the NIC photolytic degradation kinetics was performed in order to provide evidence about the quality of the drug varies with time under the influence of photolytic conditions.

In the present work, a RP-HPLC method with UV–MS detection was utilized to separate the drug from its degradation products generated during the stress test with retention time less than 10 min and to characterize the major degradants. This method could be applied for the quantitative determination of the studied drug in capsules, as well as in human plasma.

2. Experimental

2.1. Materials and chemicals

Nicardpine HCl (99.84% w/w, Lot No. 16H0190), was purchased from Sigma Chemical Company (St. Louis, MO,
USA). 0.5 M Sodium hydroxide (BDH, UK), 1 M Hydrochloric acid (Fluka Darmstadl, Switzerland), 5% Hydrogen peroxide (Riede-de Haen, Germany), 99.8% Methanol (BDH, UK), 0.01 M Triethyl amine (TEA) (Riede-de Haen, Germany) were also used. The nicardipine capsules (Pelcard capsule) contained 50 mg of nicardipine per capsule (Global Napi Pharmaceuticals, Egypt) were purchased from commercial pharmacy. Pooled blank plasma was obtained from King Fahd Military Medical Complex (Dhahran, Saudi Arabia).

2.2. Instrumentation

2.2.1. Mass spectrometry

Identification of intermediate products was performed by LC/MS. LC/MS experiments were carried out using a Waters Micromass ZQ Detector (Waters, Milford, USA) Quadrupole mass analyzer (mass range 2–2000 amu) equipped with electro-spray ionization (ESI) interface in positive mode. The needle voltage was approximately 3500 v and the cone voltage was held at approximately 60 v. MS data acquisition and analysis were performed using MassLynx® 4.0 software Micromass/C228 version 3.5.

2.2.2. Chromatographic conditions

Separations were carried out using a Waters 2695 Separation Module (Waters, Milford, USA) system consisting of a vacuum degassing module Rheodyne Model 7725 injector equipped with a 20 μL loop, a quaternary pump, column heater and variable wavelength detector. Data acquisition was performed using the MassLynx® 4.0 software Micromass/C228 version 3.5. The column was Waters Symmetry® C18 150 mm × 3.9 mm, 5 μm particles size. The column temperature was maintained at 25°C and the flow rate was 1.0 mL min⁻¹. The detector wavelength was set at 353 nm. The mobile phase consisted of 70% methanol: acetic acid containing 0.01 M triethylamine with pH 4 (Al-Ghannam and Al-Olyan, 2009).

2.2.3. Spectrophotometry

The spectrophotometric analysis was performed on double beam UV/Visible spectrophotometer V-350 (Jasco, Japan) with matched 1-cm quartz cells.

2.3. Preparation of samples

2.3.1. Preparation of standard solutions and calibration graphs

A stock solution containing 1 mg/mL of NIC was prepared in methanol. NIC stock solution was further diluted with the same solvent and then with the mobile phase as appropriate to obtain the working standard solutions ranging between 0.5 and 40 μg/mL. The peak area ratio was plotted versus the concentration of the drug (μg/mL) to get the calibration graph. Alternatively, the corresponding regression equation

<table>
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<tr>
<th>Conc. of NaOH (M)</th>
<th>Slope</th>
<th>K (min⁻¹)</th>
<th>log K</th>
<th>t₁/₂ (min)</th>
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was derived. The stock solutions were found to be stable for at least two weeks when kept at 4°C.

2.3.2. Preparation of capsules solutions

Ten capsules from NIC formulation (50 mg/capsule) were weighed and emptied then mixed well. Appropriate amount of the powdered capsules equivalent to 50 mg of the drug was transferred into 50 mL volumetric flask sonicated well with 80 mL methanol for 20 min. Then the solution was completed to the volume with the same solvent to give a stock solution of 1 mg/mL of NIC. This solution was filtered through millipore filter (0.45 µm pore size) and aliquots of this solution were diluted with the mobile phase as appropriate. The nominal contents of the capsules were calculated using either the calibration graph or the corresponding regression equation.

2.3.3. Assay of NIC in human plasma

1.5 mL aliquots of plasma were transferred into a set of centrifugation tubes. Aliquots from the drug stock solution were added so that the final concentration is in the range 5–40 µg/mL. The solution was mixed thoroughly using a vortex mixer. 0.5 mL of 0.5 M borate buffer (pH 9.0) and 5 mL of n-hexane/butanol (12:1 v/v) were added to each tube and gently mixed for 5 min. and centrifuged at 1000 rpm for 10 min. The organic phase was then transferred to a set of small beakers and evaporated under a stream of nitrogen. The residue was dissolved in 10 mL methanol. Aliquots of 30 µL were injected (triplicate) and eluted with mobile phase under the reported chromatographic conditions. A blank sample using plasma without the drug was measured simultaneously. The peak area ratio was plotted versus the concentration of the drug in µg/mL. The corresponding regression equation was derived.

2.4. Degradation conditions

2.4.1. Acid degradation

The effect of strong acid was studied in 10 mL volumetric flask by treating 10 µg/mL solution of NIC with 1 M HCl. This solution was heated at 60°C for 1 h. Samples were withdrawn (1 mL) every 10 min. and then neutralized with the corresponding

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Effect of temperature on the kinetic parameters of nicardipine (10 µg/mL) at different concentration of sodium hydroxide.</th>
</tr>
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<td>60</td>
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<table>
<thead>
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<th>Table 3</th>
<th>Effect of temperature on the kinetic parameters of nicardipine (10 µg/mL) degraded with H₂O₂ 5%.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature °C</td>
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<tr>
<td>30</td>
<td>0.0122</td>
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<tr>
<td>40</td>
<td>0.0423</td>
</tr>
<tr>
<td>50</td>
<td>0.143</td>
</tr>
</tbody>
</table>

Figure 5 Change in the absorption spectrum of nicardipine solution (10 µg/mL) in methanol after UV irradiation (0–210 min).
volume of 1 M NaOH and analyzed by the previously described HPLC/MS method.

2.4.2. Base degradation
The effect of strong alkalis was studied in 10 mL volumetric flask by treating 10 µg/mL solution of nicardipine with different concentrations of NaOH (0.1, 0.2, 0.3, 0.4 and 0.5 M). This solution was heated at (50, 60, 70 and 80°C) for 1 h. Samples were withdraw (1 mL) every 10 min. and then neutralized with the corresponding volume of 1 M HCl and analyzed by the previously described HPLC/MS method.

2.4.3. Oxidative degradation
The effect of hydrogen peroxide was studied in 10 mL volumetric flask by treating 10 µg/mL solution of nicardipine 5% with H2O2. This solution heated at (30, 40 and 50°C) for 5 h. Samples were withdraw (1 mL) every 1 h then neutralized and analyzed by the previously described HPLC/MS method.

2.4.4. UV degradation
For the kinetic study of photo degradation, the experiments were carried out at room temperature (25 ± 2°C) in a box containing a UV light system. A 4-Watt, short/long wave Ultraviolet lamp, two separate tubes (254 and 365 nm), intensity, (µW/cm²): 760/720 at 3” (Cole Parmer, USA). 10 µg/mL Nicardipine methanolic solutions was placed in a cylindrical, quartz cell of 2.4 mL capacity and 1 cm path length, and then irradiated from a source (245 nm) at a distance of 13 cm for up to 3.5 h. This solution was measured every half hour spectrophotometrically (to follow the time needed for complete drug degradation) and by HPLC/MS (to identify the intermediate compounds).

3. Results and discussion

3.1. Method development and optimization of the chromatographic conditions
Chromatographic conditions were optimized to obtain high sensitivity, good peak shape and short retention time. The separation and ionization of NIC was affected by the composition of mobile phase. Acetonitrile–water, acetonitrile–acetate, hexane–ethanol and methanol–water in various proportions were tested. In view of the response of NIC retention times, 70% methanol was the best. The ionization of NIC was increased by adding additive in the mobile phase. Therefore, triethylamine (TEA) and acetic acid were attempted to improve the response. When 30% TEA
(0.01 M) was added in the mobile phase, the peak shape of NIC was improved. The response of NIC was increased by adding acetic acid. Changing the pH over the range 3.5–5.0 was found to have no significant effect either on the retention factors of the drug and its degradation products or on the UV intensity. Based on these experiments, pH 4.0 was used. Finally, methanol/TEA (0.01 M) with acetic acid pH4 (70:30 v/v) was adopted as the mobile phase. Similarly the effect of flow rate was examined at 0.25, 0.5, 1.0 and 1.5 mL/min, the flow rate of 1.0 mL/min was used.

3.2. Purity assessment of nicardipine

Authenticity of the reference nicardipine could be assessed by several analytical techniques such as IR, NMR, Mass spectrometry and chromatographic methods. The results obtained were analyzed and matched with those of the with-submitted analytical report obtained from the manufacturer. Fig. 1 showed the electro spray mass spectrum of nicardipine. The peak of nicardipine is observed predominately as protonated molecular species [M + H+] (m/z 480). The probable structural changes in nicardipine molecule as result of degradation can be easily elucidated by matching with the initial molecular characteristics of the parent reference drug.

3.3. Stress studies

Stress testing of the drug substance can help to identify the likely degradation products, which can in turn help to establish the degradation pathways and the intrinsic stability of the molecule and validate the stability indicating powder of the analytical procedures used.

Stress degradation behavior. HPLC studies on NIC under different stress conditions suggested that NIC is susceptible to hydrolytic (acidic and alkaline), oxidative and photolytic stress conditions. The optimized HPLC–MS method was used to identify the degradation products of NIC. The HPLC–UV–MS obtained for NIC under various stress conditions are given in Fig. 2.

3.3.1. Acid hydrolysis

Nicardipine appears to be stable to acid degradation which is in agreement with acid degradation of isradipine (Abdel Ghany et al., 1998). It was observed that on heating at 60 °C in 1 M HCl, there was no degradation peak product eluted in the chromatogram. Fig. 2A shows the chromatogram after 1 h of heating the reaction mixture at 60 °C with HCl.

3.3.2. Alkaline hydrolysis

From the preliminary study conducted on the degradation of nicardipine in 0.4 M NaOH at 60 °C for 1 h, it is evident that the drug underwent decomposition. The rate of alkaline hydrolysis was fast with significant reduction in the peak area, and new peaks of the degradation products were observed. The drug was found to be highly susceptible to alkaline hydrolysis. The reaction in 0.4 M NaOH at 60 °C was so rapid that 50% of the drug was degraded within 1 h, forming degradation products at RT 6.35 and 6.92 in addition to that of remaining nicardipine RT = 8.93 min. Fig. 2B. Study of the kinetic parameters for the degradation of nicardipine involved measuring the peak height of nicardipine only.

Table 1 and Fig. 3 show the increase in the value of the reaction rate constant (K) and the decrease in the half-life time (t1/2) upon increasing the concentration of sodium hydroxide.

The combination of effect of the concentration of sodium hydroxide with the effect of time and temperature was studied. Fig. 4 shows the effect of the combination of the three factors for the concentration of 0.2 M of NaOH. Table 2 is a collection of the data representing the effect of increasing the concentration of NaOH at different temperature; the values of the slopes; reaction rate constant; half-life time and activation reaction are listed. As showed from the results the values of activation energy were 55.27–65.67 J/mol K in agreement with the corresponding values for the hydrolysis of esters (Conners et al., 1979; Ibrahim et al., 2010).

3.3.3. Oxidation

Fig. 2C shows the total ion chromatogram from the hydrogen peroxide 5% degradation after 5 h of heating the reaction mixture at 40 °C. A single major component is observed at a retention time of 7.8 min. and comprises 100% of the original sample. Table 3 listed the data of kinetic parameters for this degradation at different temperature.

3.3.4. Photolytic degradation

The most undesirable property of 1,4-DHPs from pharmaceutical point of view is their high photochemical sensitivity which can involve molecular changes leading to decrease of therapeutic effect and even some toxic effects after administration (Barand et al., 2006). In this part, the methanolic solution of nicardipine (10 μg/mL) was exposed to the UV light at 245 nm to identify the photo degradation products for the drug. As shown in Fig. 5, the exposure to UV light at wavelength = 245 nm resulted in photo induced modifications of the drug chromophore, as indicated by the significant changes of the UV spectral absorption of the solutions (irradiation time 0–210 min).

In particular a decrease of the absorption band at λ = 353 nm was observed in agreement with a previous study on related 1,4-dihydropyridine derivatives (Albini and Fasani, 1998). This decrease of absorption suggests a significant modification (aromatization) of the 1,4-dihydropyridine chromophore (Alvarez-Lueje et al., 2003). Moreover, increased absorption at λ = 280 and 310 nm can be observed and is attributed to formation of degradation products.

HPLC–UV chromatograms, acquired at 353 nm, of the photo exposed solutions Fig. 2D showed the formation of four photo degradation products at RT 4.40, 4.98, 6.06 and 7.13. The high percentage of unchanged nicardipine showed that this compound was much more stable compared with the other 1,4-dihydropyridine compounds for which no parent compound was detectable after 2 h of light exposure (Barand et al., 2006; Albini and Fasani, 1998; Alvarez-Lueje et al., 2003; Pomponio et al., 2004).
3.4. Identification of the degradation products

3.4.1. Identification of base degradation

The Chromatogram of NIC degradation showed two peaks in addition to that of NIC. It is postulated that the decomposition pathway as shown below:

Upon heating NIC in alkaline medium, it undergoes the following changes, the two ester groups attached to the dihydropyridine ring are hydrolyzed, resulting in the formation of \( \text{CH}_3\text{OH} \) (P3) which is not detectable, and N-methyl-N-(2-hydroxylethyl)benzylamine (P2).

At the same time, NIC undergoes photochemical decomposition through auto-oxidation reduction resulting in reduction of the nitro group to nitroso and oxidation of the dihydropyridine ring (P1), as previously reported (Albini and Fasani, 1998).

Compound (P1), the acid fraction (present as sodium salt) is highly polar, which have peak with high intensity more than compound (P2) or nicardipine itself (Walash et al., 2007). Fig. 6 shows the mass spectrum of degradation of nicardipine with sodium hydroxide (0.4 M) at 60 °C for 1 h, it illustrate that compound (P1) has peak intensity of 100% compared to the intensity of residual nicardipine which was 50%.

The LC/MS under the same conditions Fig. 2B shows two peaks produced from the degradation which have same intensity, therefore, it is proposed that two peaks appear to be isomers since they have the same molecular weight 353 (P2) appears in less intensity and at molecular weight 165.

3.4.2. Identification of oxidative products

As mentioned before this oxidation produced one product. Fig. 7 shows the mass spectra of nicardipine after degradation by \( \text{H}_2\text{O}_2 \) 5% at (a) \( t = 0 \) and (b) \( t = 5 \) h. It illustrates that the major compound appear at molecular weight 496. It proposed that product result from the oxidation of dihydropyridine to pyridine derivative in addition to the oxidation of the tertiary amine to the N-oxide (Milcent and Chau, 2002). The increase in molecular weight of 16 would tend to support this assignment which is equal the molecular weight of hydroxyl [M + OH]. And the chromatogram is agreed with the obtained mass spectrum where the intensity of the produced compound is equal to 75% and the intensity of residual nicardipine is equal to 50%. The proposed structure of oxidation product of nicardipine with hydrogen peroxide is suggested as follows:

3.4.3. Identification of photo degradation

In contrast to chemical degradation photo degradation shows the formation of four different products Fig. 2B. The major photo degradation product form through the conversion of the dihydropyridine ring to a pyridine ring via loss of 2H⁺. This product has been reported as the major photo degradation product from all of the dihydropyridine based calcium channel blockers studied to date (Keaneth et al., 1984; Ibrahim et al., 2010).
Figure 7: LC–MS spectrum of nicardipine solution (10 μg/mL) incubation with hydrogen peroxide for 5 h at 40°C, (a) 0 h and (b) 5 h, MS conditions: 30 V for cone voltage, 3300 V for capillary voltage, 1.5 V for extraction voltage.
Figure 8  LC–MS spectrum of nicardipine solution (10 μg/mL) in methanolic solution after different time of exposure to UV light: (a) 0 min, (b) 90 min and (c) 210 min, MS conditions: 30 V for cone voltage, 3300 V for capillary voltage, 1.5 V for extraction voltage.
The nicardipine mass spectra are shown in Fig. 8a along with a scheme of fragmentation, represented of the fragmentation pattern of all the main photoproducts. Fig. 8b shows reduction of the peak at m/z = 466 resulting from the reduction of nitro to nitrous group. Fig. 8c shows the proposed degradation products of nicardipine obtained after the exposure of a methanolic solution (10 μg/mL) to UV (245 nm) light for 210 min.

3.5. Validation of the stability-indicating HPLC–MS method

The stability-indicating HPLC–MS assay method of NIC was validated with respect to linearity, precision, accuracy and specificity as per ICH guidelines (ICH-Harmonised Tripartite Guideline, 2003).

3.5.1. Linearity

To establish linearity and range, a stock solution containing 1.0 mg/mL of NIC was diluted at different concentration and response for the NIC was found to be linear in the concentration range between 0.5 and 40 μg/mL. Linear regression analysis of the data gave the following equation:

\[ P = 0.4046 + 0.6062C \left( R^2 = 0.999 \right) \]

where \( C \) is the concentration in μg/mL, \( P \) is the peak area ratio and \( R \) is the correlation coefficient. The result shows an excellent correlation existed between the peak area ratio and concentration of the analyte.

3.5.2. Specificity and selectivity

The specificity of the method was established through study of resolution factors of the drug peak from the nearest resolving peak and also among all the other peaks (ICH-Harmonised Tripartite Guideline, 2003). The presence/absence of peaks due to excipients, impurities and degraded products was examined to study the interference from these substances in the assay of the drug. A solution containing all the capsule excipients omitting NIC was prepared and a blank plasma sample was performed under the optimum chromatographic conditions. No interfering peaks appeared in the chromatogram at the retention time of the drug or its degradation product indicating the specificity of the proposed method Fig. 9a.

3.5.3. Precision

For repeatability, a solution of 10 μg/mL was injected three times and the RSD of these three determinations was not more than 1.9% and no change in retention time of the drug. Intra-day and inter-day dilutions of 5, 15, 30 μg/mL were injected three times in the same day and in three consecutive days, respectively. The overall RSD of both intraday and interday was not more than 1.2%.

3.5.4. Sensitivity

The limit of detection (LOD) and the limit of quantification (LOQ) of NIC were determined based on signal-to-noise ratio of 3 and 10, respectively (United States Pharmacopoeia, 2007). The baseline noise was measured in a blank experiment in the region of retention time of NIC using chromatographic software. It was found that, the LOD and LOQ values were 0.11 μg/mL and 0.036 μg/mL, respectively.

3.6. Applications

3.6.1. Dosage form analysis

The proposed method was successfully applied to the assay of NIC in commercial capsules (Pelcard capsules, 50 mg). The average percentage found of different concentrations of the drug was on the average of three replicate determinations. The mean% recovery of NIC in the examined dosage form was 100.11%. High percentage recovery values revealed that the proposed method is accurate and could be adopted for routine quality control.

3.6.2. Spiked human plasma analysis

Nicardipine is rapidly absorbed from the gastro intestinal tract but is subjected to saturable first-pass hepatic metabolism. Bioavailability of about 35% has been reported following a 30 mg dose at steady state. The terminal plasma half-life is about 8.6 h (Parfitt, 1999). The method could be successfully applied to the determination of NIC in spiked human plasma Fig. 9b. The plasma calibration curve was linear with the following equation:

\[ P = 13.699 + 0.0713C \left( R^2 = 0.9991 \right) \]

where \( P \) is the peak area ratio, \( C \) the concentration of the drug in μg/mL and \( R \) is the correlation coefficient. The accuracy of the proposed method was assessed by investigating the recovery of NIC at three concentration levels covering the specified range using spectrophotometric detection. The mean% recovery of NIC was 99.04%.

4. Conclusions

A rapid, precise and accurate HPLC method has been developed for the determination of NIC either alone or in the presence of its degradation products. The method is specific and unaffected by the presence of degradants, indicating the method’s stability as a stability indicating assay of NIC, which is suitable to identify all of the degradation products. The method is also considered to be stability indicating assay for the determination of NIC in capsules without any interference from excipients. The proposed procedure, by virtue of its high sensitivity, could be applied to the analysis of NIC in spiked human plasma with no need for extraction or pretreatment steps.

Figure 9  HPLC chromatograms of (a): plasma blank; (b) NIC (10 μg/mL) in spiked human plasma.
Acknowledgments

The authors thank the Research and Developed Center in the Saudi Aramco and Regional Center for Poison Control in Dammam.

References


