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Research Article

New Sensitive HPLC Method for Evaluation of the Pharmacokinetics of New Amantadine Prodrugs as Hepatic Delivery Systems to Enhance its Activity against HCV *

Ibrahim A. Darwish, Tarek Aboul-Fadl, Nasr Y. Khalil, Ashraf M. Mahmoud, Abdul-Rahman M. Al-Obaid**

Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, P.O. Box 2475, Riyadh 11451, Kingdom of Saudi Arabia.

ABSTRACT

To improve the efficacy of amantadine (AMD) in chronic hepatitis C therapy, various prodrugs were designed and synthesized to enhance its hepatic delivery based on the incorporation of AMD into modified bile acid or cysteine derivatives. A new sensitive and selective HPLC method with fluorescence detection has been developed and validated for determination of AMD in human plasma for evaluation of the pharmacokinetics of these prodrugs. Betaxolol hydrochloride (BTX) was used as internal standard. AMD and BTX were isolated from plasma by protein precipitation with acetonitrile and derivatized by heating with 1,2-naphthoquinone-4-sulphonate (NQS) in alkaline medium (0.01 M NaOH) at 90±5 °C for 45 min. Separations were performed in isocratic mode on Nucleosil CN column (250 mm length × 3.9 mm i.d., 5 µm particle diameter) using a mobile phase consisting of acetonitrile:10 mM sodium acetate buffer (pH 3.5):methanol (20:70:10, v/v) at a flow rate of 1.5 mL min^{-1}. The derivatized samples were extracted with chloroform and reduced with 0.03% potassium borohydride. The reduced fluorescent AMD-NQS derivative was monitored at emission wavelength of 382 nm after excitation at 293 nm. Under the optimum chromatographic conditions, a linear relationship with good correlation coefficient (r = 0.9989, n = 5) was found between the peak area ratio of AMD to BTX and AMD concentration in the range of 30–3200 ng mL^{-1}. The limit of detection and limit of quantification were 6.7 and 21 ng mL^{-1}, respectively. The intra and inter-assay precisions were satisfactory; the relative standard deviations did not exceed 1.57%. The accuracy of the method was proved; the recovery of AMD from spiked human plasma were 97.51–100.95 ± 0.26–1.57%. The method had higher throughput as it involved simple sample preparation procedure and short run-time (<15 min). The results demonstrated that the proposed method would have a great value in the pharmacokinetic studies for AMD released from the synthesized produgs.

Keywords: Amantadine; Prodrugs; HPLC; pharmacokinetic studies; Plasma.

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of chronic liver disease and hepatocellular carcinoma, and is the leading indication for liver transplantation. HCV infection is a major global health problem with an estimated worldwide prevalence of 3% corresponding to about 170 million infected persons. Treatment of chronic hepatitis C continues to be an important and growing challenge. AMD has been suggested to be useful in the treatment of patients with chronic hepatitis C who have previously failed therapy with interferon α-2b. AMD costs only $20 per month, a tiny fraction of the cost of interferon, the conventional treatment for hepatitis C. Interferon costs approximately $500 per month, and must be administered for at least six months (Eldrup, 2004; Smith, 2004).

One of the goals of effective drug delivery is to control and optimize the localized release of drug at the target site and rapidly clear the non targeted fraction. The various carriers that are being proposed for hepatic-specific drug delivery are based on incorporation of the drug to modified bile acid or cysteine derivatives (Wermuth, 2003). Accordingly, improvement of the efficacy of antiviral therapy for chronic hepatitis C with AMD and minimization of its systemic side effects can be achieved through conjugation of AMD with cysteine derivatives or bile acid salts as hepatic delivery systems (Fig. 1).

For detection and kinetic study of the release of AMD from these prodrugs in the liver, a reliable sensitive analytical method was necessary. Unfortunately, AMD does not have a chromophore or fluorophore in its molecule which are the essential requirements for its

** Corresponding Author
Email: idarwish@ksu.edu.sa
Contact: +966-14677348 Fax: +966-14676220
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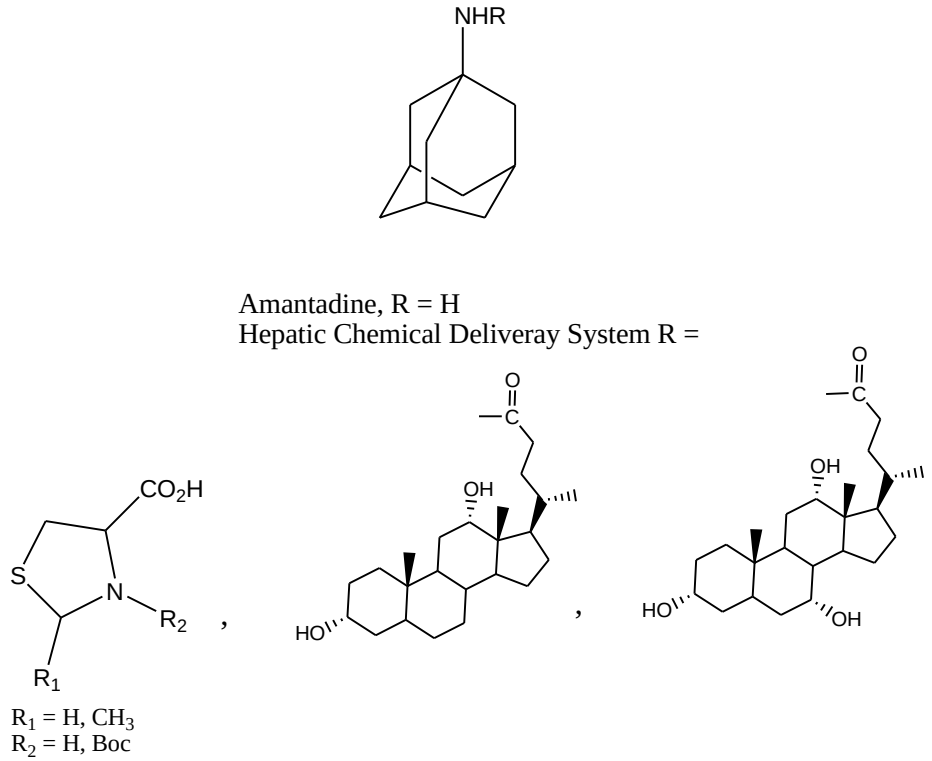

Figure 1. Amantadine (AMD) and its synthesized prodrugs.

analysis by HPLC methodology with either spectrophotometric or fluorimetric detection. Therefore, derivatization of AMD was necessary. Many derivatization methods coupled with chromatography have been established for the determination of AMD in biological matrices: TLC (Darwish, 2008), HPLC (Duh, 2005; Higash & Fuji, 2005; Higashi, 2006), GC (Leis, 2002), and capillary electrophoresis (Reichova, 2002).

1,2-Naphthoquinone-4-sulphonate (NQS) has been used as a derivatizing reagent for analysis of many compounds (Gallo-Martinez, 1998; Wang, 2005; Darwish, 2005) prior their analysis by spectrophotometric, fluorimetric, and HPLC. In our previous study (Mahmoud, 2009), we demonstrated the reactivity of AMD with NQS, and the AMD-NQS derivative was found to be highly fluorescent after its reduction with potassium borohydride (KBH_4). The present study was devoted to adopt this reaction in the development of sensitive and selective HPLC method with fluorescence detection for determination of AMD in plasma samples.

EXPERIMENTAL

Chromatographic system

HPLC apparatus consisted of a Shimadzu system (Shimadzu Corporation, Kyoto, Japan) equipped with LC-10AD VP pump with FCV-10AL VP low pressure flow control valve, SCL - 10A VP system controller, Rheodyne-7725 injection valve with 20 µL loop, SPD-10A VP,UV-visible detector, RF-10A XL fluorescence detector. The chromatographic separations were carried on Nucleosil CN analytical column (250 mm length x 4.6 mm i.d., 5 µm particle size) manufactured by Machery Nagel (GmbH, Germany). The column temperature was kept constant at 25±2 ºC. Separations were performed in isocratic mode. The mobile phase consisted of acetonitrile - 10 mM sodium acetate buffer (adjusted to pH 3.5 with 100% acetic acid) - methanol (20:70:10, v/v). The mobile phase was filtered by a Millipore vacuum filter system equipped with a 0.45 µm filter, degassed by ultrasonic bath, and by bubbling helium gas. The flow rate was 1.5 mL min^{-1}. The sample injection volume was 20 µL. The fluorescence detector was set at 293 nm as an excitation wavelength and 382 nm as an emission wavelength. The system control and data acquisition were performed by Shimadzu CLASS-VP software, version 5.032 (Shimadzu Corporation, Kyoto, Japan). The ratio of peak area of AMD to internal standard (BTX) was used for the quantitation.

Chemicals and materials

Amantadine hydrochloride (AMD; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was obtained and used as received. Betaxolol hydrochloride (BTX; Sigma Chemical Co., St. Louis, CA, USA) 1,2-Naphthoquinone-4-sulphonate (NQS; El-Nasr Pharmaceutical Chemical Co., Abo-Zaabal, Egypt). Potassium borohydride (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Human plasma samples were collected from normal healthy volunteer at King Khaled University Hospital (Riyadh, Saudi Arabia), and they were stored in deep-freezer at –20 ºC until analysis. All solvents were of HPLC grade (Merck, Darmstadt, Germany). All other materials used throughout this study were of analytical grade. Double distilled water was obtained through Nanopure II water purification system

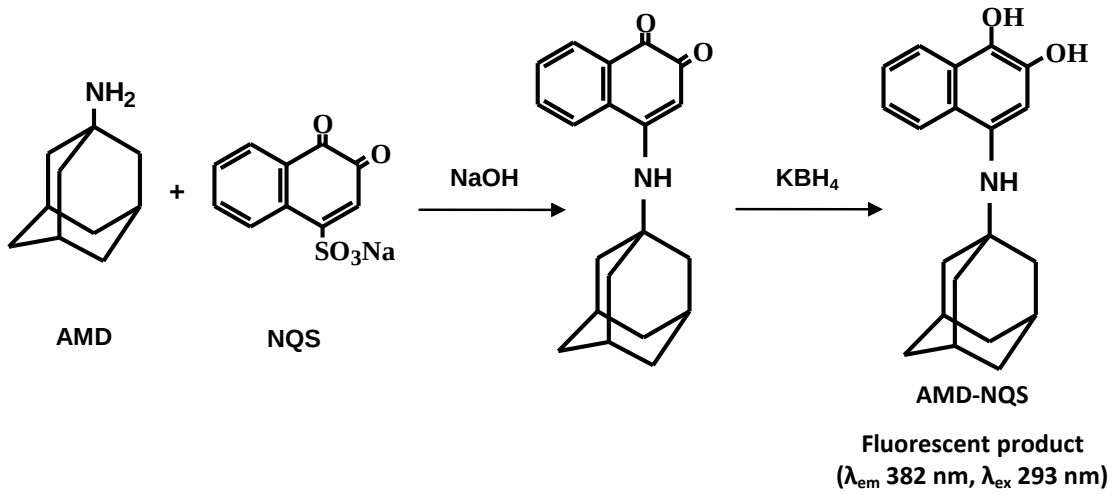

Figure 2: Scheme for the reaction pathway of AMD with NQS

(Barnstead/Thermolyne, Dubuque, IA, USA), and used throughout the work.

Preparation of solutions

AMD standard solutions.

An accurately weighed amount (25 mg) of AMD was quantitatively transferred into a 25-mL calibrated flask, dissolved in 20 mL distilled water, completed to volume with the same solvent to produce a stock solution of 1 mg mL^{-1}. This stock solution was further diluted with water to obtain working solutions in the range of 20–3200 ng mL^{-1}.

BTX internal standard solution.

Accurately weighed amount of BTX (25 mg) was quantitatively transferred into a 25-mL calibrated flask, dissolved in 20 mL distilled water, completed to volume with the same solvent to produce a stock solution of 1 mg mL^{-1}. This stock solution was further diluted with water to obtain working standard solution of 1 μg mL^{-1}.

NQS derivatizing reagent.

Accurately weighed amount of NQS (50 mg) was quantitatively transferred into a 25-mL calibrated flask, dissolved in 20 mL distilled water, completed to volume with water to produce a solution of 0.2% (w/v). The solution was freshly prepared and protected from light during use.

Preparation of plasma samples and derivatization procedure.

Series of 1.0 mL portion of plasma were spiked with AMD (20–3200 ng mL^{-1}) and BTX (100 ng mL^{-1}). The

Table 1: Summary for the optimization of variables affecting the reaction of AMD with NQS employed in the development of the proposed HPLC method with fluorimetric detection

Variable	Studied range	Optimum
NQS (%, w/v)	0.05 – 1.5	0.2
NaOH (mM)	1– 50	10
Temperature (° C)	25 – 100	90 ± 5
Time for reaction of AMD with NQS (min)	5 – 75	45
Solvent for extraction of AMD-NQS	Varying [a]	Chloroform
KBH_4 (%, w/v)	0.01 – 0.1	0.05
Time for reduction of AMD-NQS with KBH_4 (min)	2 – 20	5 [b]
pH of injected solution	1 – 7	2 [c]
Excitation wavelength (λ_{ex}, nm)	270 - 370	293
Emission wavelength (λ_{em}, nm)	300 - 470	382

[a] Solvents were carbon tetrachloride, chloroform, dichloromethane, ethylacetate, toluene, and benzene.

[b] At room temperature (25 ± 5° C).

[c] This pH was attained by diluting the reaction mixture with 0.025 M HCl ethanolic solution

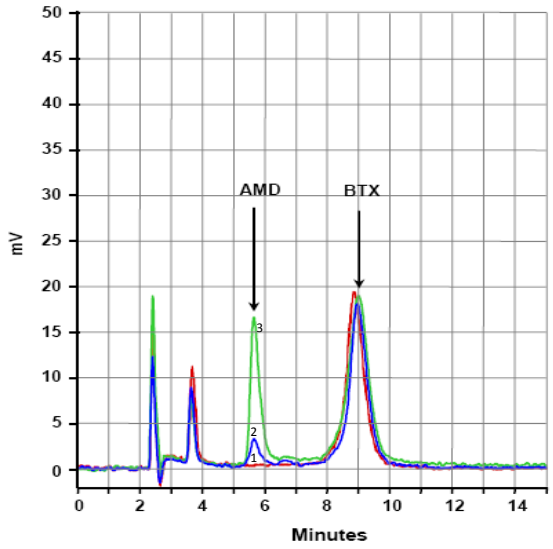

Figure 3: Representative chromatograms of blank plasma spiked with 100 ng mL^{-1} of BTX internal standard (red tracing, 1) and the same plasma sample spiked with 5 ng mL^{-1} (blue tracing, 2) and 40 ng mL^{-1} (green tracing, 3) of AMD and 100 ng mL^{-1} of BTX. mV is the detector response in millivolts.

spiked plasma samples were treated with 1.0 mL of acetonitrile and shaken by the aid of vortex for one min. The samples were centrifuged for 20 min at 13000 rpm. The supernatant (1 mL) was transferred into a test tube, and 1 mL of 0.01 M NaOH was added followed by 1 mL of NQS reagent (0.2%, w/v), and the contents of the test tubes were heated in a water bath (MLW type, Memmert GmbH, Co. Schwa bach, Germany) at 90±5° C for 45 minutes and then cooled in ice water for 2 min. KBH_4 solution (0.2 mL of 0.05%, w/v in methanol) was added and the reaction was allowed to proceed for 5 min at room temperature (25±5 °C). The solution was diluted to 5 mL with 0.025M methanolic HCl. A volume of 20 µL of the resulting solution was injected into the HPLC system.

Calibration curve using standard AMD- spiked plasma

The calibration standard samples were prepared by spiking blank human plasma with AMD to yield final concentrations of 25, 50, 100, 200, 400, 800, 1600, and 3200 ng mL^{-1}. The samples were prepared as described under *Preparation of plasma samples and derivatization procedure* and the supernatants were isolated and manipulated as described under HPLC method. An eight-point calibration curve was constructed by plotting the peak area ratio of AMD to BTX versus AMD concentration (*x*). Analysis of calibration samples at each concentration was performed in triplicate. Slope, intercept and correlation coefficient (*r*) were calculated as regression parameters by linear regression. The linear regression equation was used to calculate the concentrations of AMD in spiked plasma based on their peak-area ratios.

Table 2: Chromatographic parameters of AMD spiked in human plasma

Parameter	Value
Retention time of AMD (min)	5.67 ± 0.07
Retention time of IS; BTX (min)	8.80 ± 0.10
Capacity factor of AMD, K` (min)	1. 434
Resolution, AMD and IS peaks	5.123
Peak asymmetry	1.333
Number of theoretical plates	635
Height equivalent to theoretical plate (µm)	393

RESULTS AND DISCUSSION

Reaction involved and optimization of experimental conditions for method development

A two-step reaction was involved, the first one was the reaction of AMD with NQS in alkaline medium (with NaOH), while the second one was the reduction of the AMD-NQS derivative with KBH_4 in acidified medium (with HCl) after its selective extraction step. The excitation and emission spectra of the reduced AMD-NQS derivative were performed on RF-5301 PC spectrofluorimeter (Shimadzu, Kyoto, Japan) and it was found to exhibit the highest fluorescence intensity at λ_{em} of 382 nm after excitation at λ_{ex} of 293 nm. The scheme for the reaction is given in Fig. 2. The optimization of experimental conditions affecting this reaction was investigated by altering each reaction variable in a turn while keeping the others constant. A summary for the results of this study is given in Table 1. The optimization of the chromatographic conditions was performed in isocratic elution mode. The separation of AMD and BTX (internal standard; IS) could be adjusted by changing the composition of the mobile phase. Several experiments showed that the employment of an appropriate ratio of sodium acetate buffer solution in the mobile phase may improve the chromatographic peaks (Ding, 2003; Ding, 2007). Reciprocal variations of sodium acetate and acetonitrile contents in the mobile phase from 40:60 – 80:20 (v/v) were employed. Both AMD and BTX were eluted in very short time from the column as the aqueous content of the mobile phase decreased, however AMD was affected more than BTX. The best resolution was achieved when the content of aqueous sodium acetate:acetonitrile was 70:30 (v/v). It was found that the use of methanol in the mobile phase (at 10% on the expense of acetonitrile) improves the resolution and gives more symmetric peaks. Different concentrations of sodium acetate buffer solution at levels 10, 20, and 30 mM were tested in the mobile phase.

The results showed that the lowest concentration of 10 mM sodium acetate buffer was able to improve the shapes of the chromatographic peaks of both AMD and BTX, resulting in more symmetric peaks. The acidic pH of the mobile phase was beneficial to maintain the pH at the optimum to obtain the highest fluorescence. In

Table 3: Intra-assay and inter-assay precision and accuracy for estimation of AMD in spiked human plasma

Nominal conc. (ng mL^{-1})	Intra-assay		Inter-assay	
	Measured conc. (ng mL^{-1} ± SD)	Recovery (% ± RSD)	Measured conc. (ng mL^{-1} ± SD)	Recovery (% ± RSD)
50	48.9 ± 0.5	97.82 ± 1.04	48.8 ± 0.8	97.51 ± 1.57
100	99.4 ± 1.2	99.44 ± 1.16	100.9 ± 1.3	100.90 ± 1.25
200	201.4 ± 2.0	100.72 ± 0.97	201.1 ± 2.4	100.55 ± 1.2
400	403.8 ± 3.3	100.95 ± 0.83	397.8 ± 5.4	99.44 ± 1.36
800	800.5 ± 5.5	100.06 ± 0.69	803.8 ± 9.1	100.47 ± 1.13
1600	1590.1 ± 9.3	99.38 ± 0.59	1602.6 ± 13.0	100.16 ± 0.81
3200	3175.8 ± 8.2	99.24 ± 0.26	3193.0 ± 19.2	99.78 ± 0.60

the present study, BTX was chosen as the internal standard as it is quantitatively derivatized by NQS under the same conditions required for AMD. Most important, the derivatized BTX has the same excitation and emission fluorescence maxima, and has shown good resolution and good chromatographic profile with the applied chromatographic conditions. Another column was tested: μ-Novapak C_{18} column, 150 mm length × 3.9 mm i.d., 5 μm particle diameter (Waters, Milford, MA, USA) but the separation and resolution of the AMD and BTX was not good. Therefore, the Nucleosil CN column was selected for all the subsequent work. Under these chromatographic conditions, the run time of the sample was 12 min, and the retention times were 5.67 ± 0.07 and 8.8 ± 0.1 min (n=5) for AMD and BTX, respectively (Fig. 3).

METHOD VALIDATION

Selectivity and performance parameters

The selectivity of the method was evaluated by carrying out blank experiments in the mobile phase and different batches of blank human plasma to identify the reagent peaks and the peaks due to the derivatized biogenic plasma components. Typical chromatograms obtained from blank plasma (containing 100 ng mL^{-1} (BTX) and plasma spiked with AMD (5 and 40 ng mL^{-1}) and BTX (100 ng mL^{-1}) are shown in Fig. 3. The chromatogram showed complete separation of AMD and BTX from the reagent and endogenous plasma constituents. The chromatographic performance parameters of the AMD and BTX are presented in Table 2.

The interferences from amino acids with the assay procedures were also investigated using glycine as a model example for the amino acids. The results revealed that there was no interference coming from the amino acids after the extraction step of the AMD-NQS. The absence of interferences from amino acids was attributed to the fact that their NQS derivatives are water soluble, rather than the AMD derivatives (Pesez & Bartos, 1974; Zhao, 2006). Therefore, the extraction step of the AMD-NQS with chloroform was necessary before its reduction with KBH_4. This extraction step, in addition to its advantage in avoiding the interferences of amino acids, it was found to enhance the sensitivity as well. The improvement of the sensitivity by extraction for AMD-NQS prior its reduction with KBH_4 was attributed to the reducing of the background signal without affection the analytical signal.

Linearity and sensitivity

Under the above optimum conditions, linear relationship with good correlation coefficient (r = 0.9989, n = 5) was found between the peak area ratio of AMD to BTX (Y) versus AMD concentration (X) in the range of 30–3200 ng mL^{-1}. The limits of detection (LOD) and limits of quantitation (LOQ) were computed (ICH guidelines, 2005) using the formula: LOD or LOQ = κSDa/b, where κ = 3 for LOD and 10 for LOQ, SDa is the standard deviation of the intercept, and b is the slope. Calculations on 5 replicate experimental injections, the LOD and LOQ were 6.7 and 21 ng mL^{-1}.

Precision

Intra-assay precision was studied at three concentration levels (50, 200, and 1600 ng mL^{-1}) of AMD and 100 ng mL^{-1} of the internal standard (BTX). Three aliquots were prepared from each concentration level, and injected into the HPLC system. The RSD of the ratios between AMD and BTX peak areas were 1.04, 0.97, and 0.59% for 50, 200, and 1600 ng mL^{-1}, respectively. The RSD calculated for the values of the peak areas corresponding to the IS during the study was 3.54% (n = 18). The RSD calculated for the values of the retention time corresponding to the chromatographic peaks was 0.75 and 0.82% for AMD and IS, respectively.

Inter-assay precision was carried out on five different days at the same concentration levels for spiked plasma samples freshly prepared daily. The RSD of the ratios between AMD and BTX peak areas were 1.57, 1.20, and 0.81% for 50, 200, and 1600 ng mL^{-1}, respectively. The RSD calculated for the values of the retention time corresponding to the chromatographic peaks was 1.25 and 1.48% for AMD and IS, respectively.

Accuracy

The accuracy was determined by analyzing the quality control plasma samples spiked at different concentration levels (50, 100, 200, 400, 800, 1600 and 3200 ng mL^{-1}) of AMD and 100 ng mL^{-1} of the IS. The values of the ratios between AMD and IS were then interpolated

in the linear regression equation computed in the linearity study, to calculate the experimental concentration values. The accuracy was presented as a percent recovery ± RSD. The accuracy was 97.51-100.95 ± 0.26–1.57% (Table 3) indicating the accuracy of the proposed method.

Robustness and ruggedness

In order to measure the extent of the method robustness, the most critical parameters were interchanged while keeping the other parameters unchanged. The chromatographic parameters were interchanged within the range of 1–10% of the optimum recommended conditions. The studied parameters were: the pH of the composition of the mobile phase, percentage of methanol, concentration and pH of sodium acetate buffer, pH of the derivatization reaction mixture, and column temperature. The chromatographic profile including: capacity factor (k`), retention time (Rt), peak asymmetry, resolution, and column efficiency were calculated and compared with those of the system suitability (Table 2). The results revealed that the method was robust for these small changes in methanol content, concentration of sodium acetate in the mobile phase, and its pH in the range of 3.2–3.7. The increase in the column temperature generally decreased the k` values, and the column temperature has to be maintained at 25±2 °C. The ruggedness of the method was evaluated by applying the recommended analytical procedures on the same HPLC system by three of authors of the study (independently on different days) on the analysis of series of AMD samples. The RSD values of the k`, Rt, and peak areas obtained from the three operators were not more than 2%.

CONCLUSIONS

The study described the development of HPLC method with fluorescence detector for determination of AMD after its pre-column derivatization with NQS. The sample preparation procedure was very simple and robust as it did not involve liquid-liquid extraction of AMD from plasma samples. It was based on only protein precipitation with acetonitrile followed by the derivatization reaction. The derivatized sample was directly injected into the HPLC system. The chromatographic separation was based on a reversed phase mechanism carried out under isocratic elution mode for only a 12-min run time. The analytical results demonstrated that the proposed method is suitable for the accurate quantification of AMD in human plasma at concentrations as low as 21 ng mL^{-1}, and has a wide linear range. The simple procedure involved in the sample preparation and the short run-time added the property of a higher throughput to the method.

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