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Article

A Sensitive and Rapid Determination of Ranitidine in Human Plasma by HPLC with Fluorescence Detection and its Application for a Pharmacokinetic Study

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A novel precolumn derivatization reversed-phase high-performance liquid chromatography method with fluorescence detection is described for the determination of ranitidine in human plasma. The method was based on the reaction of ranitidine with 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole forming yellow colored fluorescent product. The separation was achieved on a C_{18} column using methanol–water (60:40, v/v) mobile phase. Fluorescence detection was used at the excitation and emission of 458 and 521 nm, respectively. Lisinopril was utilized as an internal standard. The flow rate was 1.2 mL/min. Ranitidine and lisinopril appeared at 3.24 and 2.25 min, respectively. The method was validated for system suitability, precision, accuracy, linearity, limit of detection, limit of quantification, recovery and robustness. Intra- and inter-day precisions of the assays were in the range of 0.01–0.44%. The assay was linear over the concentration range of 50–2000 ng/mL. The mean recovery was determined to be 96.40 ± 0.02%. This method was successfully applied to a pharmacokinetic study after oral administration of a dose (150 mg) of ranitidine.

Introduction

Ranitidine HCl, *N*-(2-[(5-(dimethylaminomethyl)furan-2-yl) methylthio]ethyl)-*N*-methyl-2-nitroethene- 1,1-diamine HCl (RAN) (Figure 1), is an inhibitor of gastric acid secretion that is used in the treatment of gastric and duodenal ulcers. It competitively inhibits the action of histamine on the H_2 receptors of parietal cells (1, 2).

Certain analytical methods have been published for the determination of RAN in biological fluids. Among these methods, high-performance liquid chromatography (HPLC) (3–13), high-performance thin layer chromatography (HPTLC) (14), supercritical fluid chromatography (15), capillary electrophoresis (16) and polarography (17) have been utilized for the determination of RAN.

In addition, several methods have also been reported for the determination of RAN in pharmaceutical preparations. These articles include voltammetry (18), titrimetry and uv-spectrophotometry (19), kinetic spectrophotometry (20), flow injection potentiometry and spectrophotometry (21), flow-injection extraction-spectrophotometry (22), near-infrared reflectance spectroscopy (23) conductimetry (24), HPLC (25, 26), HPTLC (27) and capillary electrophoresis (28).

A new fluorimetric HPLC method was developed in this study. The method is based on the derivatization of RAN with 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F). The method was optimized, fully validated and successfully applied to the pharmacokinetic study.

Experimental

Reagents

RAN (salt of hydrochloride) was supplied from Sigma (Steinheim,Germany). Ranitab 150 mg film tablets were obtained from a local pharmacy. Standard lisinopril, which was used as internal standard (IS), was kindly supplied from Sanovel (Istanbul, Turkey). NBD-F was purchased from Fluka (Buchs, Switzerland). Other chemicals were provided from Merck (Darmstadt, Germany). All solvents were of analytical grade.

Apparatus and chromatographic conditions

Fluorescence spectrum of the RAN-NBD derivatized compound was recorded by utilizing a Model RF-1501 spectrofluorimeter from Shimadzu (Kyoto, Japan). The liquid chromatographic system was a Shimadzu Liquid Chromatography (Kyoto, Japan) consisting of a Model LC 20 AT solvent delivery system with an SIL-20AHT autosampler with a 5 μL loop and a RF-10AXL fluorescence detector. The analytical column was an Inertsil C_{18} column (150 mm × 4.6 mm i.d., 5 μm) with a guard column (4 mm × 3 mm i.d., Inertsil) packed with the same material. The mobile phase was comprised of methanol–water (60:40 v/v). Analyses were run at 40°C at a flow rate of 1.2 mL/min. The fluorimetric detector was set at 458 and 521 nm for the excitation and emission wavelengths, respectively.

Standard solution preparation

Stock standard solutions of RAN and IS were prepared in methanol at a concentration of 100 μg/mL and stored at +4°C. Ten μg/mL for RAN and 1 μg/mL for IS solutions were prepared from the stock solutions by using methanol. Standard solutions were prepared in a 0.5 mL volume for human plasma to yield final concentrations of 50 and 2000 ng/mL. The NBD-F solution was freshly prepared at 0.2 mg/mL in methanol. Borate buffer, pH 9.0, was prepared by dissolving 0.620 g boric acid and 0.750 g potassium chloride in 100 mL of water. The pH was adjusted with 0.1 M sodium hydroxide solution and the volume was made up to 200 mL with water.

Sample preparation and derivatization

All of the frozen plasma samples were thawed just before the experiment. Calibration curves were constructed by adding various amounts (25–1000 ng) of RAN to aliquots (0.5 mL) of drug-free human plasma and a fixed amount (20 ng) of the IS, and 2.0 mL of 0.1 M NaOH were added. The solution was

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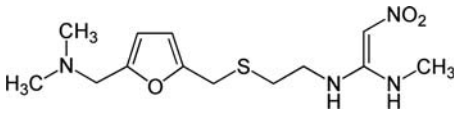

Figure 1. Chemical structure of RAN

vortex-mixed and then 3.0 mL of ethyl acetate was added. The solution was mixed once more for 5 min and centrifuged for 35 min at 4500 rpm. The organic layer was transferred into another clean Eppendorf tube and dried under a stream of N_2 in a 40°C water bath. Borate buffer (100 μL) and NBD-F solutions (100 μL) were added to the residue. The solution was mixed vigorously and kept in a water bath at 70°C for 10 min. The tube was then cooled and 100 μL of 1M HCl was added to avoid the existence of NBD-OH. The content of tube was extracted three times with 2 mL of chloroform. A 5 mL aliquot was evaporated to dryness. The residue was dissolved in 1.0 mL mobile phase and filtered through a 0.2 μm membrane filter. Then the sample was injected into the HPLC system.

Method validation

The optimum HPLC conditions were investigated and resolution (Rs), theoretical plate number (N), capacity factor (k') and tailing factor (T) were found as criteria for system suitability testing. The proposed method was validated according to the International Conference on Harmonization (ICH) Guidance Documents Testing (29). The system precision, accuracy, linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, robustness and specificity parameters were analyzed.

Precision and accuracy

Intra-day and inter-day precision and accuracy were determined at low, medium and high concentrations of RAN by replicate analyses of plasma samples. The accuracy and precision of the method was expressed by relative mean error (RME%) and by relative standard deviation (RSD%), respectively.

Linearity

The IS technique that was utilized to provide the repeatability and calibration curve of RAN-NBD derivative was constructed by employing the ratio of peak normalization (rPN) against concentration. In the statistical evaluation of calibration parameters such as slope (a) and intercept (b), their standard deviations (SD) and correlation coefficient (r) were calculated.

Limit of detection and limit of quantification

LOD and LOQ were calculated from the equation of [(SD of intercept)/(slope of regression) by multiplying 3.3 and 10, respectively.

Recovery

The extraction recovery for plasma at three different concentrations of RAN was determined. Known amounts of RAN were

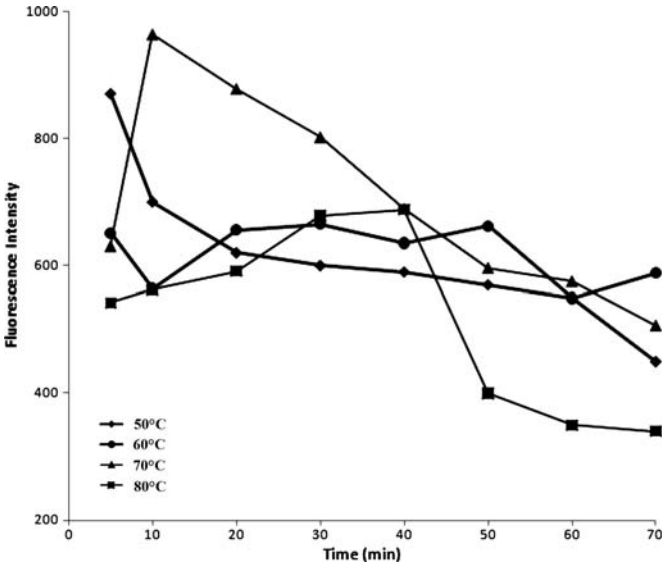

Figure 2. Effect of time and temperature on the reaction of RAN with NBD-F

added to the drug-free plasma. After the derivatization chromatography processes, the peak areas were compared to the peak areas obtained from the aqueous solutions of RAN at the same concentration.

Robustness

The robustness of the HPLC method was determined by analyzing the samples under a variety of conditions such as small changes in the percentage of mobile phase combination (methanol and water) flow rate of mobile phase, pH of buffer, excitation and emission wavelengths.

Pharmacokinetic study

RAN was administered in a single dose of 150 mg to 40 year-old healthy female volunteers. Further blood samples were drawn into ethylenediaminetetraacetic acid tubes at 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 10, 12 and 24 h after administration. Samples were centrifuged and plasma was separated and stored at −20°C until analysis. The maximum plasma drug concentration (C_{max}) and the time to reach maximum plasma drug concentration (t_{max}) were observed directly from the concentration-time data. The apparent elimination half-life ($t_{1/2}$) was calculated as $0.693/k_e$. The area under the plasma concentration−time curve (AUC) was calculated by the trapezoidal rule.

Results and discussion

Derivatization

Because RAN has no native fluorescence, its direct fluorimetric determination of RAN is not possible without derivatization. NBD-F has a highly labile fluoride atom that is displaceable by nucleophilic groups such as thiol, primary and secondary amines. Some studies have been performed subjecting the

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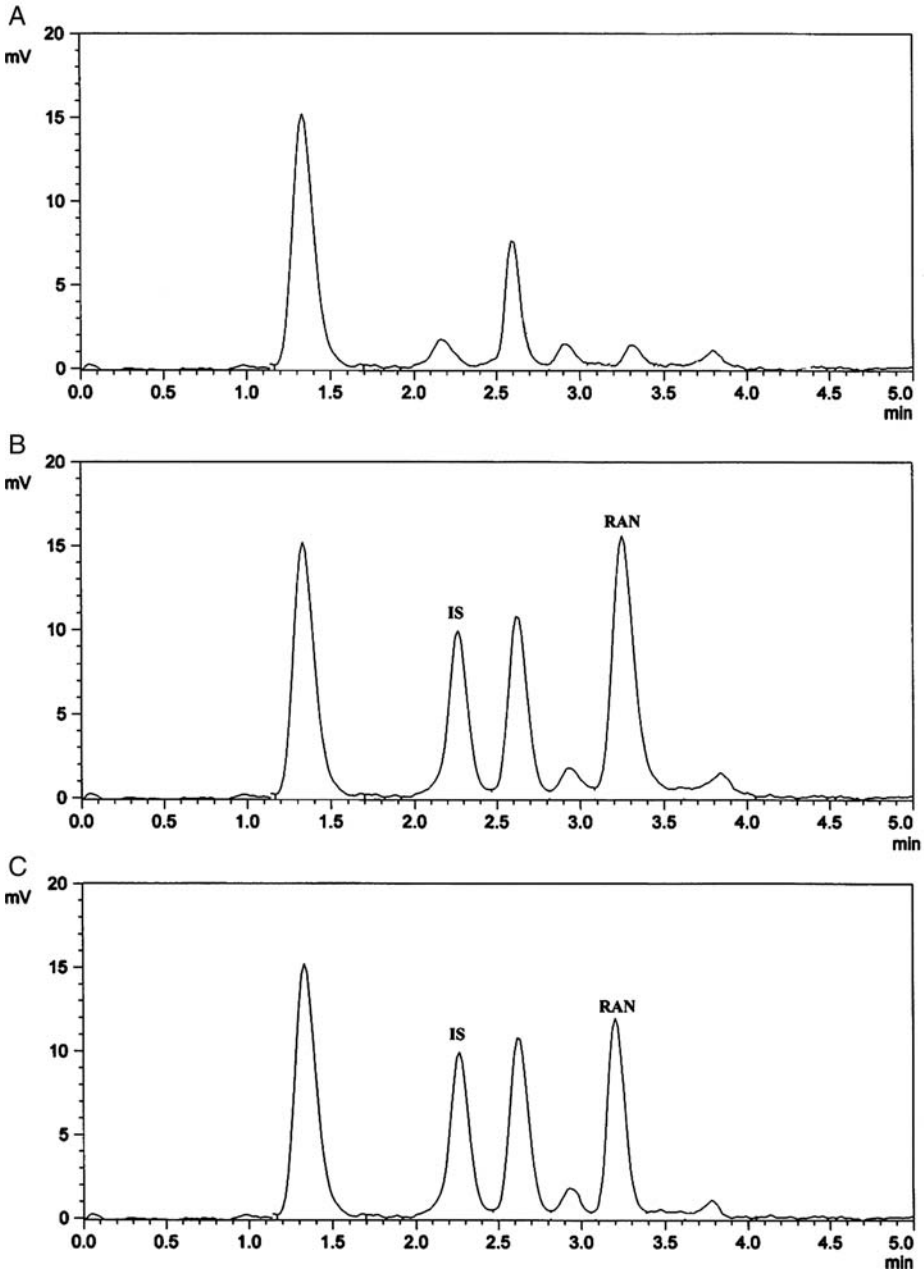

Figure 3. (A) Chromatograms of drug-free plasma with NBD-F; (B) plasma spiked with RAN-NBD (1000 ng/mL) IS-NBD (40 ng/mL); (C) plasma sample obtained at 2.5 h after oral administration of 150 mg of RAN from a healthy volunteer.

derivatization of certain pharmaceuticals to NBD-F employing spectrofluorimetry, HPLC and capillary electrophoresis (30).

The preliminary experiments showed that the secondary amino groups of RAN associated with NBD-F in an alkaline buffer medium yielded a highly yellow fluorescent product that exhibited the highest fluorescence intensity at 521 nm after excitation at 458 nm.

Optimum association conditions were investigated initially. The pH was examined in the range of 8–10 pH in borate buffer. Because the fluorescence is developed only in alkaline medium, the study of the pH was restricted to the range of 8–10. The highest fluorescence intensity for RAN was obtained by using borate buffer at pH 9.

Table I
Results of System Suitability Evaluations

Parameters	IS-NBD	RAN-NBD	Optimum
t_R	2.24	3.25	–
k′	5.80	2.45	>2
T	0.91	1.09	1.10 > T > 0.9
R_s	3.77	2.25	>2
N	8094.49	11263	>2000

The influence of different heating temperatures and times were studied by using a water bath at four different temperatures between 50–80°C for RAN-NBD derivatives (Figure 2).

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Table II
Intra and Inter-Day Precision and Accuracy of Method for RAN-NBD Compound

Intra-day ($n = 6$)

First day			Second day			Third day			Inter-day($n = 18$)		
Found (ng/mL)	RSD%	RME%	Found (ng/mL)	RSD%	RME%	Found (ng/mL)	RSD%	RME%	Found (ng/mL)	RSD%	RME%
50.3	0.19	+0.60	50.2	0.22	+0.40	50.2	0.18	+0.40	50.2	0.29	+0.47
250.2	0.20	+0.08	250.4	0.12	+0.16	250.8	0.06	+0.32	250.5	0.13	+0.19
2000.6	0.02	+0.03	2000.7	0.26	+0.04	2000.8	0.02	+0.08	2000.7	0.10	+0.04

The highest and constant yield for derivatization was obtained at 70°C and a reaction time of 10 min.

Chromatographic conditions

An isocratic HPLC method employing fluorescence detection was utilized for the determination of RAN. To develop a rugged and suitable HPLC method, different combinations of methanol–water (50:50, 60:40, 70:30, 75:25, 80:20, 85:15, v/v) mobile phases were tested. The variation of the mobile phase leads to considerable changes in the chromatographic parameters; the most appropriate mobile phase combination was found to be a mixture of methanol–water (60:40, v/v) in the previously mentioned conditions. This system was found to be suitable to retain RAN-NBD derivative in the column. This was observed to retain a short time and a good column resolution (the peak symmetry and a reduction in the tailing factor). RAN-NBD derivative appeared at 3.24 min. An IS was then carried out for the previously mentioned chromatographic system and lisinopril was an appropriate compound that appeared at 2.25 min (Figure 3). The chromatogram was not at all affected from the matrix of the plasma in the studied conditions.

Theoretical plates, capacity factor (k'), tailing factor (T), resolution (Rs), and theoretical plate number (N) are also determined. The results obtained are all within acceptable limits (Table I).

Liquid–liquid extraction

A liquid–liquid extraction method was chosen. A large group of extraction solvents such as methanol, ethanol, *n*-hexane, ethyl acetate, dichloromethane, chloroform and 2-propanol were tried to develop a single step liquid–liquid extraction procedure for a good recovery. Ethyl acetate was found to be a good extracting solvent for sample preparation because it resulted in a clean chromatogram.

Method validation

Precision and accuracy

Intra- and inter-day precision and accuracy were calculated by measuring the amount of RAN in spiked plasma injecting six times of each concentration level (50, 250 and 2000 ng/mL RAN). The results from the precision and accuracy of the method in human plasma are listed in Table II.

Linearity, LOD and LOQ

Calibration plots were constructed by plotting the concentration against RAN-NBD compound to IS peak area ratio. The variation showed a good linearity in the range of 50–2000 ng/mL in matrix of plasma.

Table III
Statistical Calibration Results for RAN-NBD Derivative ($n = 6$)

Parameters	Value
Concentration range (ng/mL)	50–2000
a ± SD	$0.0013 \pm 1.1 \times 10^{-4}$
b	0.1556
r	0.9995
LOD	6.0
LOQ	18.0

Table IV
Recovery Results of RAN-NBD from Plasma

Amount of RAN added (ng/mL)	Amount of RAN found (ng/mL) (mean ± SD, $n = 6$)	Recovery (%)	RSD (%)
50	47.72 ± 0.01	95.44	0.02
500	481.75 ± 0.11	96.35	0.02
2000	1948.43 ± 0.14	97.42	0.01

LOD and LOQ values for RAN-NBD were calculated to be 6. and 18.0 ng/mL. These values are lower than those obtained by many other reported methods (4, 7, 10–13, 25). The statistical results of linearity and calculation of the LOD and LOQ are presented in Table III.

Recovery

The efficiency of the extraction procedure was tested for the three samples at three concentrations (50, 500 and 2000 ng/mL). Very good results were obtained in the range of 95.4–97.4% (Table IV). These values are much better than those obtained by using other methods (4, 10).

Robustness

The robustness is a measurement of the method capacity to remain unaffected by small, but deliberate, variations in method parameters, and was studied by testing influences of small changes in mobile phase composition. Mobile phase composition had very strong influence on peak retention time and separation. All critical separations were achieved with the indicated minimum baseline resolution (Table V).

Specificity

The specificity of the method was tested by comparing the chromatograms of blank plasma samples from three

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Table V
Robustness Results for the Proposed Method (500ng/mL RAN-NBD derivative)

Conditions	Mean ± SD (ng/mL)	RSD (%)
Optimum conditions (60:40, v/v, 1.2 mL/ min)	500.3 ± 0.30	0.06
Mobile phase		
61:39, v/v	500.4 ± 0.22	0.04
59:41, v/v	500.3 ± 0.31	0.06
Flow-rate		
1.3 mL/ min	500.4 ± 0.22	0.04
1.1 mL/ min	500.3 ± 0.31	0.067
pH		
9.1	500.2 ± 0.28	0.06
8.9	500.3 ± 0.30	0.06
Wavelengths (λ_{ex}, λ_{em} nm)		
459 nm, 522 nm	500.4 ± 0.21	0.04
457 nm, 520 nm	500.3 ± 0.30	0.06

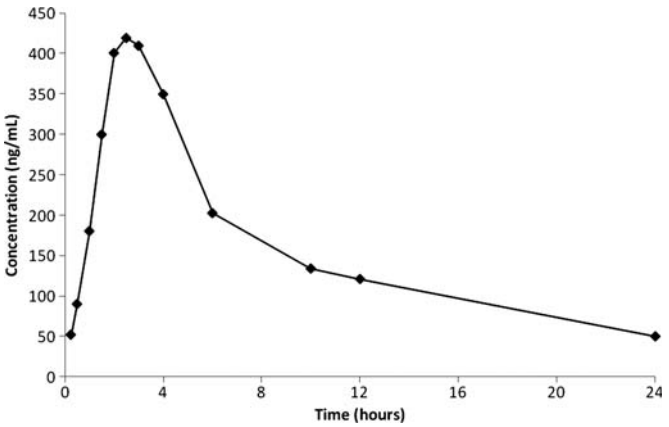

Figure 4. Plasma concentration–time profile of RAN in a healthy volunteer after a single oral administration of 150 mg

Table VI
Pharmacokinetic Parameters for RAN in Healthy Volunteers

t_{max} (h) $t_{1/2}$ (h)	C_{max} (ng/mL)	AUC $_{0-24}$ (h ng/mL)
2.5 2.9	419.6	2533.1

humans with those of plasma spiked with RAN and IS. No interferences were detected at the retention times of RAN and IS.

Application to pharmacokinetic study

The method was applied to determine the plasma concentration of RAN after an oral administration of 150 mg RAN to one volunteer. After oral administration of 150 mg RAN, the median of t_{max} and the mean of C_{max} were found to be 2.5 h and 419.6 ng/mL, respectively. Plasma concentrations declined with $t_{1/2}$ of 2.9 h. The $AUC_{0\text{-}24}$ value obtained was 2533.1 ngh/mL (Figure 4, Table VI). Because the bars of SD values for each point are very low, they do not appear on the pharmacokinetic profile.

Conclusion

A novel and sensitive fluorescence HPLC method for RAN after pre-column NBD-F derivatization in human plasma was developed and validated. Precolumn derivatization was found to overcome some problems such as tailing peaks and low detector sensitivity by the formation of amines, which can be more easily analyzed by HPLC.

In this study, the purpose of the derivatization reaction is to raise sensitivity, and thus, the possibility of working in low concentrations. The advantages of liquid–liquid extraction method include good extraction recovery and a simple and less time-consuming procedure. The recovery percentage of RAN is high (3, 9); derivatization and extraction processes do not take much time. Besides, according to the other methods, the retention time is quite short (3, 4, 6-9, 13), Particularly in comparison with previous methods (4, 7, 10–13, 25). The method proposed here shows several advantages because of better LOD and LOQ and the employment of a methanol–water system instead of buffered systems (3–13). Therefore, flushing of the column is not required.

As a conclusion, the method developed here can be proposed for both routine pharmaceutical analysis and monitoring of the plasma concentrations of RAN in bioavailability and bioequivalence studies.

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