

# Sensitive determination of ranitidine in rabbit plasma by HPLC with fluorescence detection

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## Abstract

A sensitive high-performance liquid chromatographic method for determination of ranitidine (RAN) in rabbit plasma is described. The method is based on liquid–liquid extraction, labeling with dansyl chloride and monitoring with fluorescence detector at 338 nm (ex)/523 nm (em). Plasma samples were extracted with diethyl ether alkalized with 1 M sodium hydroxide. Ephedrine HCl (EPH-HCl) was used as internal standard. Both, RAN and EPH were completely derivatized after heating at 60 °C for 10 min in sodium bicarbonate solution (pH 9.5). The derivatized samples were analyzed by HPLC using Agilent Zorbax Extended C<sub>18</sub> column (150 mm × 4.6 mm i.d.) and mobile phase consists of 48% acetonitrile and 52% sodium acetate solution (0.02 M, pH 4.6). The linearity of the method was in the range of 0.025–10 µg/ml. The limits of detection (LOD) and quantification (LOQ) were 7.5 ± 0.18 and 22.5 ± 0.12 ng/ml, respectively. Ranitidine recovery was 97.5 ± 1.1% (*n* = 6; R.S.D. = 1.8%). The method was applied on plasma collected from rabbits at different time intervals after oral administration of 5 mg/kg ranitidine HCl.

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**Keywords:** Ranitidine; Ephedrine; Dansyl chloride; Plasma; Fluorescence; HPLC

## 1. Introduction

Ranitidine [*N*-[2-[[[-5-[(dimethylamino)methyl]-2-furanyl]methyl]thio]ethyl]-*N'*-methyl-2-nitro-1,1-ethenediamine] is a histamine H<sub>2</sub>-receptor antagonist and used for short-term treatment of active duodenal ulcer [1–4]. Derivatives of dansyl chloride (DAN-Cl) have been widely used for HPLC determination of a large variety of amines [5] however; it is not previously used for derivatization of RAN. Vinas et al. reported an HPLC method for the determination of ranitidine and its metabolites with fluorescence detection [6]. The method was based on the reaction of the drugs with sodium hypochlorite, giving rise to primary amines that reacted with *o*-phthalaldehyde and 2-mercaptoethanol to form fluorescent products. The limit of quantification of this method is 100 ng/ml. Several high-performance liquid chromatographic methods with UV detection have been described for the determination of ranitidine in rabbit urine and plasma [7–10]. The reported

limits of detection and quantification of these HPLC-UV methods were in the range of 3–5 and 15–20 ng/ml, respectively. The British Pharmacopoeia cited an HPLC method with UV detection at 230 nm for purity testing of RAN in bulk form [11]. The literature review showed many authors used dansyl chloride for derivatization of EPH and subsequent HPLC-fluorescence analysis [12–14]. Besides, Aymard et al. described HPLC method for determination of ephedrine with fluorescence detection after derivatization with 9-fluorenylmethyl chloroformate [15]. Several extraction methods of ranitidine from plasma have been reported. Liquid–liquid extraction procedures after deproteinization have been described in different reports using 60% perchloric acid [7], acetonitrile [16], acetonitrile with potassium carbonate [10] or dichloromethane [17]. In addition, several solid-phase extraction procedures have been described for purification and recovery of RAN from plasma [8,18–21].

In the present paper, a sensitive HPLC separation method of RAN after derivatization with dansyl chloride and subsequent measurement using fluorescence detector is described. In this work, a liquid–liquid extraction procedure is described. Ranitidine was recovered from plasma without need for depro-

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teinization of plasma. Ephedrine was used as internal standard and derivatized in the same manner as RAN.

## 2. Experimental

### 2.1. Chemicals and reagents

All solvents were of HPLC grade, Merck, Darmstadt, Germany. All other materials were of analytical grade. 5-Dimethylaminonaphthalene-1-sulphonyl chloride (dansyl chloride) purchased from Sigma–Aldrich, Germany. Ranitidine hydrochloride was obtained as gift from Jamjoom Pharmaceuticals, Jeddah, Kingdom of Saudi Arabia. Ephedrine HCl was purchased from Fluka, Chemie, GmbH, Buchs, Switzerland. Rabbit blood samples were collected from ear marginal vein of rabbits weighing 1–2.5 kg.

### 2.2. Equipment

The HPLC system used consisting of an Alliance Waters separations module 2695, waters 2996 Photodiode array detector, and Waters 2475 multi  $\lambda$  fluorescence detector (Milford, MA, USA). HPLC system control and data processing was performed by Empower software (Build 1154, Waters). Screw capped V-shaped vials, 300  $\mu$ l, with PTFE liners were used (Alltech, GmbH, Unterhaching, Germany). Heating oven (Heraeus, Kendro, Hanau, Germany) was adjusted at 60 °C. Calibrated digital micro-transfer pipettes 5–250  $\mu$ l, Brand, Wertheim, Germany were used.

### 2.3. Chromatographic conditions

Analytes were separated on an Agilent Zorbax Extend-C<sub>18</sub>, 150 mm  $\times$  4.6 mm, 80 Å, 5  $\mu$ m (Agilent Technologies, Palo Alto, CA, USA). The analytical column was protected with pre-column: Agilent Zorbax Extend-C<sub>18</sub>, 4.6 mm  $\times$  12.5 mm, 80 Å, 5  $\mu$ m (Agilent Technologies, Palo Alto, CA, USA). The mobile phase was programmed to deliver 48% acetonitrile and 52% sodium acetate (0.02 M adjusted to pH 4.5 with acetic acid) with a flow rate of 1.0 ml/min. The fluorescence detector was set at 338 and 523 nm as excitation and emission wavelengths, respectively. The column was washed after each ten repetitive injections with mobile system consisted of acetonitrile:methanol:water (40:40:10, v/v/v) for 30 min.

### 2.4. Derivatization reagent, internal standard solution and bicarbonate solution

Ten milligrams of dansyl chloride (DAN-Cl) was accurately weighed into 10-ml volumetric flask, dissolved in about 5 ml acetonitrile, and diluted to the volume with acetonitrile. A volume of 50  $\mu$ l from this solution was used for derivatization.

Internal standard solution was prepared by dissolving 20 mg of ephedrine HCl (EPH-HCl) in 100 ml water. One milliliter from this solution was further diluted to 10 ml to give a final concentration of, 20  $\mu$ g/ml. A volume of 100  $\mu$ l from this solution was used as an internal standard.

Sodium bicarbonate (0.1 M) solution was prepared in water and adjusted to pH 9.5  $\pm$  0.1 with 1 M sodium hydroxide and checked by calibrated pH-meter.

### 2.5. Standard solutions and quality control samples

Ranitidine hydrochloride (RAN-HCl) 0.2 mg/ml, aqueous solution was prepared and used for preparation of calibration and quality control samples. Appropriate dilutions in water were prepared from this stock solution to obtain calibration standards in the range of 0.1–40  $\mu$ g/ml and quality control samples, 0.025, 0.5 and 10.0  $\mu$ g/ml.

Dilutions for calibration standards were prepared daily, while quality control (QC) samples were prepared in plasma, divided in small aliquots and stored at –20 °C until use. A sample volume of 1 ml of QC sample was extracted and analyzed at time intervals of 0, 10 and 30 days using EPH-HCl as an internal standard.

### 2.6. General derivatization procedure

A volume of 50  $\mu$ l from RAN-HCl standard solution and 100  $\mu$ l EPH-HCl solution was transferred to an autosampler glass vial using digital micropipette. A volume of 100  $\mu$ l bicarbonate solution (pH 9.5) and 50  $\mu$ l DAN-Cl solution was added. The vial was capped, swirled, and left to stand at 60 °C in hot air oven for 10 min. The vial was then cooled, and a volume of 10  $\mu$ l was injected for HPLC analysis.

Blank experiments were carried out to identify any underivatized RAN or EPH by HPLC by using water instead of sample solution and acetonitrile instead of DAN-Cl solution.

### 2.7. Calibration curves

#### 2.7.1. Standard solutions for calibration curve

Six standard solutions of RAN-HCl were prepared spanning the range 0.1–40  $\mu$ g/ml. A volume of 50  $\mu$ l from each RAN-HCl concentration with 100  $\mu$ l of EPH-HCl solution was derivatized as described under general derivatization procedure (Section 2.6). A volume of 10  $\mu$ l was injected for HPLC analysis. The percentage of peak area ratio of RAN-DAN to EPH-DAN was plotted versus the RAN concentration in ng/ $\mu$ l of the final dilution. The calibration curve was constructed using a least-square regression equation for the calculation of the slope, intercept and correlation coefficient. This curve was used for calculation of response factor and % recovery of the drug from plasma.

#### 2.7.2. Calibration curve of plasma extract

A volume of 0.2 ml of rabbit plasma was spiked with 50  $\mu$ l standard RAN-HCl (0.1–40  $\mu$ g/ml). Each sample was extracted and derivatized as described under Section 2.8. The calibration curve was produced by linear regression of percentage of peak area ratios (RAN-DAN to EPH-DAN) against their respective concentrations in ng/ $\mu$ l of the final dilution. The regression line was used to calculate the concentrations of RAN-HCl recovered from spiked rabbit plasma based on the % of peak area ratios.

## 2.8. Extraction and derivatization of plasma

A volume of 50  $\mu\text{l}$  from each strength of standard RAN-HCl solution was spiked in 0.2 ml rabbit plasma to give a serial concentration covering the range of 0.025–10  $\mu\text{g}/\text{ml}$ . A volume of 0.2 ml of spiked rabbit plasma was transferred to 10-ml centrifuge tube and mixed with 100  $\mu\text{l}$  of 1 M NaOH. The samples were extracted by vortexing three successive times with 5 ml of diethylether for 5 min. The organic phases were separated after centrifuge at 5300 rpm and evaporated to dryness under gentle stream of nitrogen gas. A volume of 50  $\mu\text{l}$  DAN-Cl solution was added and vortexed for 1 min, then a volume of 100  $\mu\text{l}$  of EPH-HCl solution (internal standard, 20  $\text{ng}/\mu\text{l}$ ) and 150  $\mu\text{l}$  sodium bicarbonate solution (pH 9.5) were added and vortexed again for 1 min. Then the tube content was transferred to 0.3-ml autosampler vial, kept at 60  $^{\circ}\text{C}$  for 10 min, cooled and a volume of 10  $\mu\text{l}$  was injected for HPLC analysis. Blank experiment was carried out using 0.2 ml control plasma spiked with 50  $\mu\text{l}$  water instead of RAN solution.

## 2.9. Real rabbit plasma extraction and derivatization

Six male New Zealand white rabbits weighting between 1.0 and 2.5 kg were included in this study. The rabbits were kept under standard animal housing conditions. A dose of 5  $\text{mg}/\text{kg}$  of ranitidine HCl soluble in 5 ml water was introduced orally. Blood samples were withdrawn from the marginal ear vein at time intervals of 0, 15, 30, 1, 2, 4 and 6 h. The blood samples were immediately centrifuged at 5300 rpm for 20 min and plasma was aspirated and kept frozen at  $-20^{\circ}\text{C}$  until analysis. A volume of 0.2 ml of each rabbit plasma sample was transferred to 10-ml centrifuge tube, extracted, derivatized and analyzed as described under Section 2.8.

## 2.10. Calculations

The peaks eluted at 9.0 and 15.7 min are corresponding to dansylated ranitidine (RAN-DAN) and dansylated ephedrine (EPH-DAN), respectively. The amount of RAN in the injected solution (as  $\text{ng}/\mu\text{l}$ ) was obtained from the calibration curve (of spiked plasma extract) using percentage of peak area ratio of RAN-DAN to EPH-DAN. The calculated amount of RAN-HCl was multiplied by 1.5 to get the concentration as  $\mu\text{g}/\text{ml}$  of plasma, considering the % recovery factor of 100/97.5.

## 3. Results and discussion

The UV spectrum of RAN in acidic pH characterized by two  $\lambda_{\text{max}}$  at 227 and 313 nm, and it has  $A$  (1%, 1 cm) value of 499 [22]. This absorptivity does not confer enough sensitivity for determination of RAN in biological fluids. It was necessary to develop a more sensitive procedure for determination of RAN in plasma. This method was applied for quantification of ranitidine in rabbit plasma after extraction and derivatization with dansyl chloride. The derivatized product was monitored with fluorescence detector at 338 nm (ex)/523 nm (em). The proposed reaction pathway is shown in Fig. 1. It is not confirmed yet if the reaction stoichiometry of DAN-Cl to RAN is one to one or two to one, respectively. The reaction pathway is proposed to proceed via formation of one-to-one mol on the more basic nitrogen.

### 3.1. Chromatographic variables

To obtain the best overall chromatographic conditions, the mobile phase was optimized by examining the effect of pH, content of acetonitrile or methanol and the effect of counter ions (including tetrabutylammonium bromide and *n*-hexane sulfonic

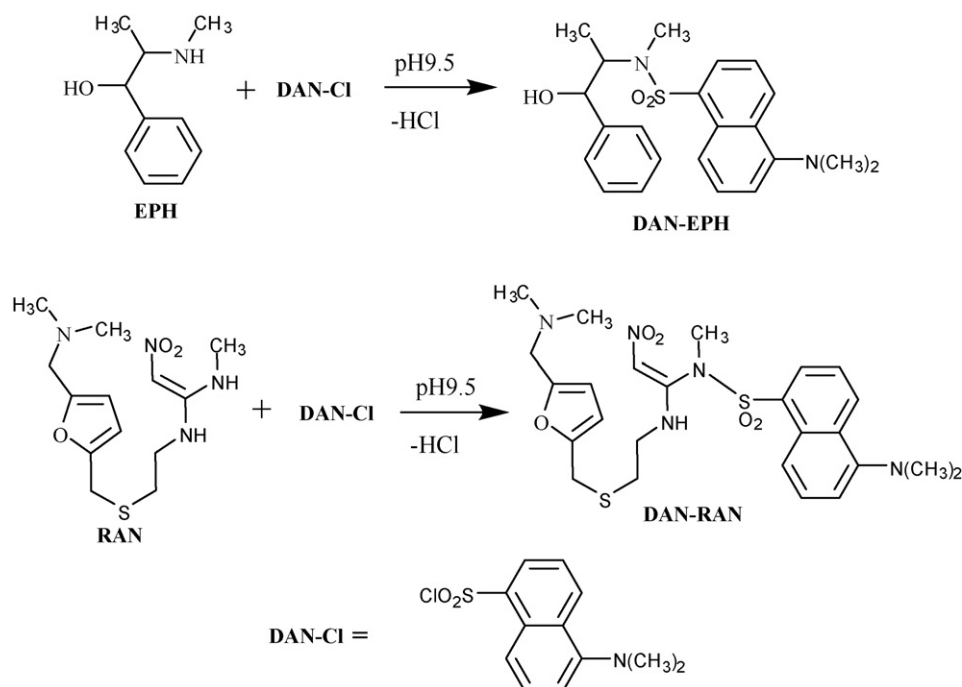


Fig. 1. Proposed reaction pathway of DAN-Cl with both RAN and EPH.

Table 1  
Chromatographic parameters<sup>a</sup> of dansylated RAN and EPH ( $n=6$ )

Name	Rt (min)	Area (R.S.D.)	Width (min)	$K'$	$\alpha$	$R$ (R.S.D.)	As	$n$
RAN-DAN	9.043	2,668,740 (1.20)	0.82	5.03			1.15	8200
EPH-DAN	15.740	3,595,580 (0.88)	1.48	9.49	1.89	12.14 (0.14)	1.13	8502

<sup>a</sup>  $K'$ , capacity factor;  $\alpha$ , selectivity coefficient;  $R$ , USP resolution; As, peak asymmetry; and  $n$ , USP plate count.

acid sodium salt). Other chromatographic variables were investigated including column temperature, column type and flow rate. The optimal chromatographic conditions were achieved as described above. The fluorescence response of RAN was decreased or abolished upon using any of the above-mentioned counter ions. Acetonitrile was used to give better resolution and symmetric peaks; however, upon using methanol, band broadening with more retardation was observed.

In this study, a simple extraction method with internal standard solution in diethylether was used for sample preparation. Initially, a liquid–liquid extraction process was performed using chloroform, ethyl acetate, *n*-hexane, or dioxane, on basified spiked plasma samples with 0.1 ml triethylamine (100%) or 1 M NaOH. These solvents or solvent mixtures give low % recovery (<20%) with irregular data. The efficiency of derivatization was interrupted upon using triethylamine for basification. Several deproteinization solvents such as acetonitrile, methanol, or 60% perchloric acid were also tested for sample preparation. No more trials were performed using perchloric acid since it has been reported to give a % recovery of  $95 \pm 4.5$  [7]. The results showed that deproteinization by acetonitrile gave poor recovery (54%) due to strong protein binding of the drug. These liquid–liquid extraction procedures have shown low % recoveries and many interfering chromatographic peaks due to plasma endogenous substances. Thus, diethylether was selected as it gave 97.5% recovery in addition to minimal peaks belonging to endogenous plasma substance.

In the present study, ephedrine hydrochloride was chosen as internal standard because it is quantitatively derivatized by DAN-Cl under the same conditions required for RAN. More important, the dansylated EPH could be measured at the same excitation and emission fluorescence maxima as DAN-RAN, and it has shown good resolution and good chromatographic profile with the applied chromatographic conditions. Two C<sub>18</sub> columns were tried including; Agilent Zorbax-C<sub>18</sub> Eclipse XDB (4.6 mm i.d.  $\times$  25 cm length, 5  $\mu$ m particle diameter) and  $\mu$ -Bondapak C<sub>18</sub> column (3.9 mm i.d.  $\times$  300 mm length, 5  $\mu$ m particle diameter). Both columns have shown band broadening and tailed peaks. The best chromatographic separation was achieved upon using Agilent Zorbax Extended C<sub>18</sub> column (150 mm  $\times$  4.6 mm i.d.).

### 3.2. Optimization of derivatization procedure

For the time course of derivatization, the reaction time was set at 0, 2, 5, 10, 20, 25, or 30 min at 60 °C. Both RAN and EPH were completely derivatized in bicarbonate solution pH 9.5 after 10 min standing at 60 °C in hot air oven. The amount of DAN-Cl required for complete derivatization was optimized using dif-

ferent strengths. The amount of DAN-Cl used was enough for complete derivatization of both RAN and EPH extracted from plasma. This was ensured by observing the underivatized drugs by UV detection, in addition to the acceptable values of the calibration curve. The reagent concentration was investigated using spiked rabbit plasma. Relatively high concentrations of RAN and EPH were used to be detectable by HPLC-UV at 235 nm. The amount of reagent used was enough for derivatization of both RAN and EPH in the concentration range studied. However, the amount of  $7.7 \pm 0.7$   $\mu$ g of reagent was enough for complete derivatization of both RAN ( $\leq 10.0$   $\mu$ g) and EPH ( $\leq 2.0$   $\mu$ g). These relatively high concentrations of RAN and EPH were derivatized and analyzed by HPLC-UV and HPLC-fluorescence detection (HPLC-FLD) for monitoring the underivatized and derivatized forms. Also this relatively high concentration of DAN-Cl was applied to derivatize the drug, In St and the extracted biogenic plasma substances. Subsequently, the same samples were diluted 10 times before HPLC-FLD/HPLC-UV analysis.

Among bicarbonate solution, pH 9–10, no significant difference of peak area was observed, and thus pH 9.5 was selected. The same results were obtained upon using 100 or 200  $\mu$ l of bicarbonate solution with total volume of reaction mixture of 300  $\mu$ l. The peak area of the derivatized RAN and derivatized EPH reached a maximum after 5 min and tends to decrease after 60 min. Therefore, the derivatization time of 10 min was selected. The % recovery of RAN from plasma sample treated with triethylamine was 54%; however, samples treated with 1 M NaOH have shown higher % recoveries (97.5).

The plasma residue was treated first with DAN-Cl solution and vortexed to dissolve ranitidine base then treated with internal standard and bicarbonate solution. This sequence of mixing steps affords precise data. The method is claimed to be robust, since small changes of the chromatographic or derivatization procedure did not affect the method precision or recovery. Also, Nova-Pak C<sub>18</sub> 60 Å column (150 mm  $\times$  3.9 mm i.d., 4  $\mu$ m particle diameter, from Waters, Milford, Ireland) was tried. This column gave the same separation profile as well as Extended C<sub>18</sub> column.

### 3.3. Selectivity, precision and performance parameters

The precision of % recoveries of RAN and EPH from plasma were calculated from the corresponding calibration curve of each standard RAN-HCl and standard EPH-HCl. The % recoveries of RAN-HCl and EPH-HCl from plasma were,  $97.5 \pm 1.1\%$  ( $n=6$ ; R.S.D. = 1.8%) and  $100 \pm 0.07\%$  ( $n=6$ ; R.S.D. = 0.02%), respectively. The blank experiments were carried in aqueous and plasma extract solutions to identify the



Table 2  
Within- and between-day precision and accuracy for determination of RAN in spiked plasma

Nominal concentration (ng/ml)	Within-day			Between-day		
	Observed concentration <sup>a</sup> (ng/ml)	CV <sup>b</sup> (%)	Relative error (%)	Observed concentration <sup>a</sup> (ng/ml)	CV <sup>b</sup> (%)	Relative error (%)
100	98.65 (2.12)	2.15	1.35	99.4 (2.23)	2.24	0.60
500	494 (1.22)	0.25	1.20	500.5 (2.20)	0.44	0.10
5,000	4895 (2.53)	0.05	2.10	4963 (2.44)	0.05	0.74
10,000	9987 (3.07)	0.03	0.13	9991 (2.72)	0.03	0.08

<sup>a</sup> Mean (standard deviation),  $n = 6$ .

<sup>b</sup> CV = coefficient of variation.

reagent peaks and the peaks due to derivatized biogenic materials. A representative chromatogram (Fig. 2) showed complete separation of both DAN-RAN and DAN-EPH from reagent and endogenous plasma constituents. The chromatographic performance parameters of the RAN and EPH are presented in Table 1.

### 3.4. Linearity and sensitivity

A linear HPLC response of peak areas for RAN was observed over the range, 0.016–6.66 ng/ $\mu$ l of the injected solution which was equivalent to 0.025–10  $\mu$ g/ml plasma. The squared regression coefficient was 0.9994, and the values of slope and intercept were 18.45 and 0.45, respectively. The response factors of RAN and EPH were 73,600 and 58,240, peak area unit per each 1 ng

injected. The limit of quantification (LOQ) was estimated by satisfying two criteria: the signal to noise (S/N) ratio is not less than 10 and the percent relative standard deviation (%R.S.D.) of five replicate injections of the LOQ solution is less than 6%. The LOQ of RAN was  $22.5 \pm 0.12$  ng/ml of plasma, and the limit of detection (LOD) value was  $7.5 \pm 0.17$  ng/ml plasma. Thus, this method is more sensitive and selective than any previously reported procedure.

### 3.5. Precision and accuracy

Within- and between-day precision and accuracy were evaluated by analyzing six replicates of quality control samples at four different concentrations of RAN (Table 2). Precision was expressed as the coefficient of variation, though accuracy was presented as a percent error (relative error), [(observed concentration – nominal concentration)/nominal concentration]  $\times$  100]. Within- and between-day relative standard deviations were less than 2.25%. Accuracy was within 2.1% when compared with nominal concentrations. The results indicate that the method is reliable, reproducible and accurate.

### 3.6. Recovery and stability

The calibration curve of RAN prepared in water was used for calculation of the % recovery from plasma. Recovery was calculated by comparing the peak areas obtained from the quality control (QC) samples to those from the standard solutions containing the same amount of RAN. The mean recoveries of RAN from plasma performed at three representative concentrations of 0.1, 0.5, 5.0 and 10.0  $\mu$ g/ml were 97.5% [relative standard deviation (R.S.D.) = 1.8%], 97.9% (R.S.D. = 1.4%), 97.8% (R.S.D. = 1.7%) and 98.9% (R.S.D. = 2.1%), respectively.

The stability of RAN in rabbit plasma was investigated through three freeze–thaw cycles of the QC samples during the storing period of 1 month at  $-20^\circ\text{C}$ . Ranitidine was considered stable in rabbit plasma after three freeze–thaw cycles at concentration of 0.1, 0.5 and 10.0  $\mu$ g/ml. The mean concentrations following this storage period were 98.1% (R.S.D. = 1.9%), 97.5% (R.S.D. = 2.2%) and 97.1% (R.S.D. = 2.3%) of RAN concentrations in freshly prepared samples, respectively. Results of the stability experiments indicated that RAN in the plasma samples was stable for at least 1 month when stored at  $-20^\circ\text{C}$ .

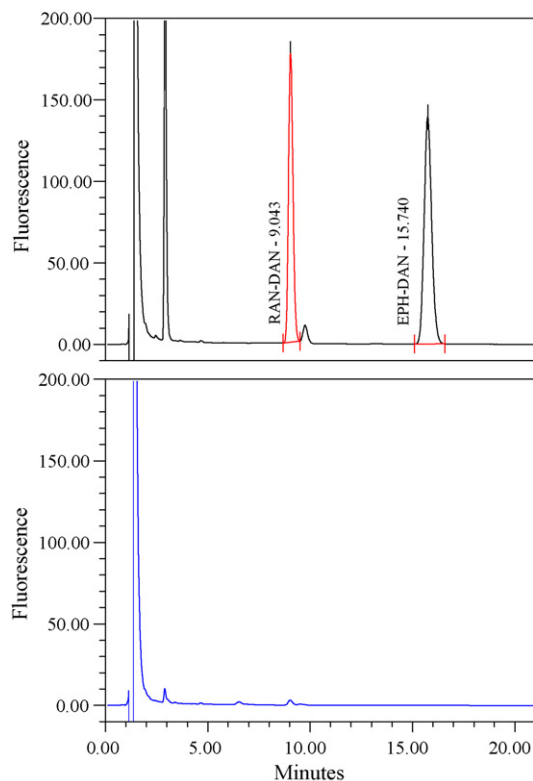


Fig. 2. Representative chromatograms of dansylated ranitidine extracted from rabbit plasma after 30 min from oral administration of 10 mg ranitidine HCl (upper, 3.6  $\mu$ g/ml) and dansylated blank rabbit plasma extract (bottom).

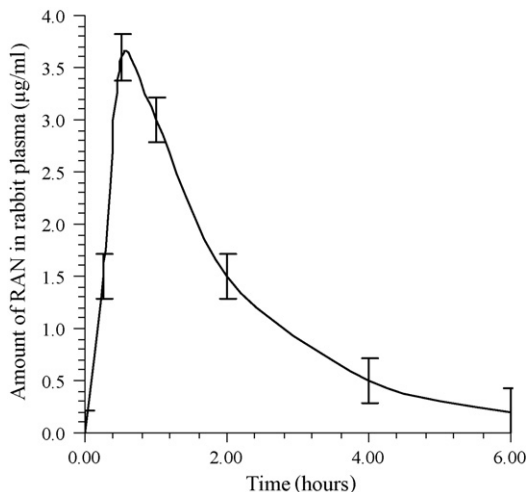


Fig. 3. Ranitidine plasma concentration vs. time determined following oral administration of 5 mg/kg single dose ( $n = 6$ , solid line for the average data).

### 3.7. Application of the method

The analyzed samples collected from New Zealand white rabbit weighing 2.0 kg (administered 10 mg RAN-HCl powder) have shown no interference from the endogenous plasma constituents. The average amounts of RAN found in plasma samples collected at time intervals of 0.25, 0.50, 1.0, 2.0, 4.0 and 6.0 h were  $1.23 \pm 0.9$ ,  $3.63 \pm 0.6$ ,  $3.00 \pm 2.1$ ,  $1.27 \pm 1.8$ ,  $0.53 \pm 2.4$  and  $0.20 \pm 1.7$  µg/ml, respectively (Fig. 3). Typical chromatograms of dansylated ranitidine extracted from rabbit plasma after 30 min from oral administration of 10 mg ranitidine HCl and dansylated blank rabbit plasma extract is presented in Fig. 2. The peak observed at 3.10 min was observed in most real samples but was not confirmed since we do not have standard metabolites.

## 4. Conclusion

The described HPLC method is simple, specific and sufficiently sensitive for the analysis of RAN in rabbit plasma after derivatization with dansyl chloride. The simple extraction procedure for sample preparation and HPLC analyses with only a 20-min run time enhanced the efficiency of the procedure. The method required only 200 µl of plasma, making it suitable for the combined pharmacokinetic and pharmacodynamic studies

of ranitidine, in which small volumes of plasma samples for HPLC assay are required. The developed method is also characterized by enough selectivity, precision, no interference with the endogenous plasma peaks and high % recovery of RAN from plasma.

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