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Il Farmaco 56 (2001) 291–296

IL FARMACO

Use of 7-fluoro-4-nitrobenzo-2-oxo-1,3-diazole (NBD-F) for the determination of ramipril in tablets and spiked human plasma

A.A. Al-Majed *, J. Al-Zehouri

Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, PO Box 2457, Riyadh 11451, Saudi Arabia

Received 19 June 2000; accepted 20 November 2000

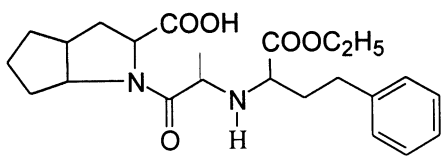
Abstract

Ramipril, as a secondary amine compound, reacts with 7-fluoro-4-nitrobenzo-2-oxo-1,3-diazole (NBD-F) producing the corresponding fluorescent NBD-ramipril. According to this fact, spectrophotometric and fluorimetric methods for the determination of ramipril were developed. The effect of these parameters on the reaction product were carefully studied to optimize reaction conditions. The relationship between the absorbance at 465 nm and the concentration was found to be linear over the range 1–10 μg/ml. Moreover, the fluorescence intensity was also found to be directly proportional at the concentration over the range of 20–100 ng/ml at 530 nm after excitation at 465 nm. The proposed procedure was successfully applied to the determination of ramipril in both tablet dosage form and in plasma. Spectrophotometric determination of ramipril tablets yielded a percentage recovery of 98.66 ± 0.38, while the percentage recovery of spectrofluorimetric determination of ramipril in spiked human plasma was $99.08 \pm 1.11\%$. The results obtained are in good agreement with those obtained by the reference method. No interference could be observed from the co-administered drug (hydrochlorothiazines).

Keywords: Ramipril; Plasma; Tablets; Fluorimetry; Colorimetry; ACE; NBD-F

1. Introduction

Ramipril, 2-[*N*-[(*S*)-1-ethoxycarbonyl-3-phenylpropyl]-L-alanyl-(1*S*,3*S*,5*S*)-2-azabicyclo[3,3,0]octane-3-carboxylic acid, is an orally active inhibitor of angiotensin converting enzyme (ACE). It is an antihypertensive drug used to treat all forms of high blood pressure [1–5].



Ramipril

Ramipril has no official analytical method in pharmacopoeias; nevertheless, reviewing the literature revealed that several methods have been reported for the quantitative and qualitative determinations of ramipril in dosage forms and in biological fluids. Spectrophotometry [6], GC [7,8], HPLC [6,9–11], radioimmunoassay [12], atomic absorption spectroscopy [13] and voltammetric methods [14] were reported.

As much as the GC and HPLC methods were sensitive and gave accurate results, they are also time consuming, cumbersome and require highly sophisticated instrumentation, which are therefore not suitable for routine analysis for large number of samples. The spectrophotometric method is much less sensitive than the fluorimetric method, particularly the analysis of the drug in biological fluids. The radioimmunoassay is faster, more specific and generally more sensitive than other methods. However, some require the use of radioactive isotopes and are therefore subject to specific regulations and require expensive reagents and equipment. Therefore, it is clear that there is a need for a simple economic as well as sensitive method for routine analysis of ramipril.

As spectrofluorometry is among the most sensitive methods of analysis, it has been chosen for developing

* Corresponding author.
E-mail address: almajed99@yahoo.com (A.A. Al-Majed).

0014-827X/01/$ - see front matter
PII: S0014-827X(01)01070-9

a method of analysis of ramipril. This drug contains an amino group, which makes it a suitable candidate for derivatization by fluorogenic reagent such as 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F). This reagent has been proposed as a replacement for chloro analog, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), as the former is 50–100 times more reactive as a fluorogenic reagent for amines [15].

Therefore, NBD-F was chosen as the fluorogenic reagent for ramipril in an attempt to develop an alternative method that is sensitive and simple enough for the determination of the drug in dosage form and in biological fluids.

2. Experimental

2.1. Apparatus

UV–Vis spectrophotometer, Shimadzu, UV-160, IPC was used for spectrophotometric measurements.

Spectrofluorimeter, Kontron SFM 25 equipped with a 150-W xenon high-pressure lamp was used for measuring fluorescence intensity (relative fluorescence).

2.2. Reagents and materials

An authentic sample of ramipril was purchased from Merck Co. (FRG), Tritace tablet labeled to contain 5 mg of ramipril/tablet were obtained from commercial sources (Russ. Production under License from Hoechst, Lot. No. 103952).

Plasma was obtained from King Khaled University Hospital, Riyadh, Saudi Arabia.

(NBD-F) reagent was purchased from Sigma Aldrich Chemie (GmbH), and was prepared to 1 mmol/l of NBD-F in ethanol.

A standard solution of ramipril was prepared in methanol to contain 1 mg/ml, then further diluted with the same solvent as appropriate. Phosphate buffer (pH 7), was prepared using 0.066 M potassium dihydrogen phosphate and 0.066 M disodium hydrogen phosphate.

2.3. Procedure

2.3.1. Calibration graphs

Standard series of ramipril were prepared by transferring suitable amounts of ramipril solution into 10 ml screw-capped test tubes. It was diluted with methanol to 1 ml, followed by addition of 2.6 ml of phosphate buffer solution. Then 0.2 ml of NBD-F solution was added. The test tubes were screw-capped and heated in a water-bath at 70°C for 10 min. The tubes were then cooled and 0.2 ml of 2 M HCl solution was added; the solution was then diluted with methanol to 10 ml. The absorbance of the solution was measured after 10 min at 465 nm; the fluorescence intensity was measured at 530 nm with excitation at 465 nm against a blank solution.

The blank solution was prepared exactly as the sample solution, but without the addition of the drug as follows: 1 ml of methanol, 2.6 ml of buffer solution and 0.2 ml of NBD-F were added to the test tubes. The tubes were screw-capped and heated in a water-bath at 70°C for 10 min. The tubes were then cooled and 0.2 ml of 2 M HCl solution was added; the solution was then diluted with methanol to 10 ml.

2.3.2. Procedure for the tablets

Twenty tablets were weighed and powdered, then a quantity of the powder equivalent to 5 mg of ramipril was transferred into a 50-ml volumetric flask containing 20 ml distilled water. The flask was shaken for 15 min and diluted to 50 ml with the same solvent, and then filtered. One milliliter of the filtrate was transferred into a 10-ml volumetric flask and diluted with distilled water to the mark. One milliliter of the resulting solution was transferred to a test tube and used as test solution; then the protocol described in Section 2.3.1 was followed. The absorbance of the solution was measured at 465 nm. The content of the tablets was determined either from the calibration graph or using the corresponding regression equation.

For quantitative measurements, the final concentrations of the NBD-F reagent and ramipril were 2×10^{-5} and 2.4×10^{-6} mmol, respectively.

2.3.3. Procedure for plasma

The spiked plasma (100 μl) was diluted to 1 ml with a mixture of diethylether and *n*-pentane (3:2), then 30 μl from 5% hydrogen carbonate solution was added. The resulting solution was mixed well and centrifuged at 3000 rpm for 5 min. Exactly 100.0 μl of the organic solvent was transferred into the screw-capped tube; then the protocol described in Section 2.3.1 was followed, measuring the fluorescence of solution at 530 nm after excitation at 465 nm. The concentration of ramipril in plasma was calculated either from a previously plotted calibration graph or using the corresponding regression equation.

3. Results and discussion

Ramipril, like other ACE inhibitor substances, absorbs light weakly in the UV region. Therefore, direct UV-spectrophotometry cannot be used for its analysis. However, being a secondary aliphatic amine, it can react with NBD-F via the formation of the Meisenheimer complex to give a yellow-colored fluorescence. The fluorimetric reaction of the drug with NBD-F exhibits its maximum absorbance in the visible region

at 460 nm (Fig. 1) and its highest fluorescence intensity at λ_{ex} of 465 nm and λ_{em} of 530 nm (Fig. 2).

The various experimental parameters affecting the development and stability of the reaction product were extensively investigated to determine the optimal conditions for the assay procedure. Such parameters, which were changed individually, include the pH of the solution, buffers and time of heating.

The influence of pH on fluorescent intensity was studied using different pH values. Maximum fluorescence intensity occurs at approximately pH 7.0 (Fig. 3) in 0.66 M phosphate buffer. Other buffers having the same pH value, such as borax solution and borate buffer, were also tried and compared with the phosphate buffer. The maximum fluorescent intensity was obtained using either phosphate or borate buffer. However, the result using borate buffer was non-reproducible.

The influence of temperature on color development was also studied (Fig. 4). The color intensity increased on increasing the applied temperature up to 70°C. The effect of the reaction time on the reaction course was studied by measuring the corresponding absorbance at constant temperature for different periods of time. It was found that the optimum reaction time is 10 min. The results are shown in Fig. 5. The color product was found to be stable for 1 h, which permits the application of the proposed method to the determination of the studied sample either in pure form, biological fluids or their dosage forms.

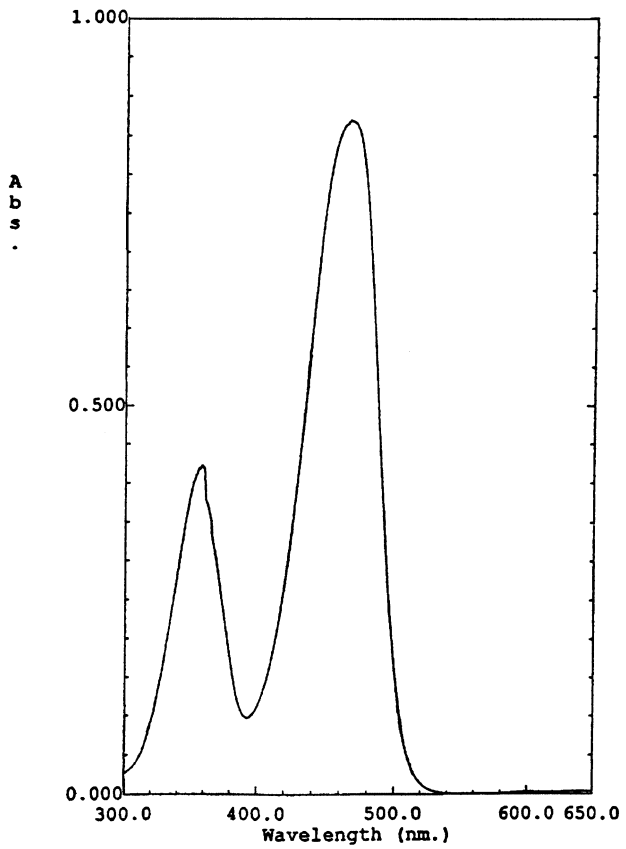
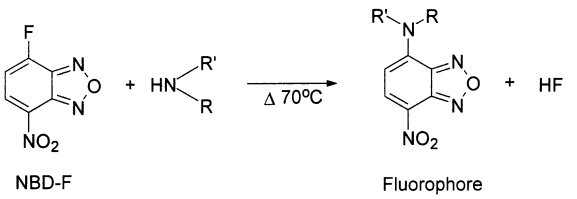


Fig. 1. UV–Vis scan for (9.6×10^{-9}) mol/ml ramipril after reaction with NBD-F in methanol solvent.

The hydrolysis product of NBD-F (NBD-OH, 4-hydroxy-7-nitrobenzo-2-oxa-1,3 diazole) can cause problems by interfering with the fluorescence product. The fluorescence of the hydrolysis product is quenched by decreasing the pH of the reaction medium to less than 1 [16]. Therefore, the reaction mixture was acidified prior to measurement of the fluorescence intensity.

Furthermore, the molar ratio of NBD-F to ramipril in the reaction mixture was studied according to Job's method of continuous variation [17]. Utilizing equimolar solution of ramipril and NBD-F $(3 \times 10^{-1}$ mmol), the reaction stoichiometry was found to be a good approximation 1:1 ratio (drug/reagent), confirming that one molecule of ramipril reacts with one molecule of NBD-F. Accordingly, the reaction was postulated to proceeded as proposed in the following scheme.



NBD-F Fluorophore

Under the previous reaction conditions, linear correlation between the absorbance and the concentration of the ramipril was found to be in the range 1–10 μg/ml, with molar absorptivity value of 60,600 l/mol cm. The relationship between absorbance, A, at 465 nm and concentration, C (1–10 μg/ml) was expressed by the following equation: $A = 0.1475C + 0.0011$, with a correlation coefficient of 0.9989 at 95% confidence limits showing excellent linearity. The RSD (%) of this method was 0.46% which indicate that the proposed method is sufficiently precise.

Similarly, the fluorescence intensity was found to be linear over a concentration range of 20–100 ng/ml. Linear regression analysis of the results gave the following equation:

$$F(\%) = 0.4C + 0.233$$

where C is the concentration in μg/ml and $F(\%)$ is the % relative intensity. The RSD (%) of the spectrofluorimetric method was found to be 1.69% showing good precision.

To assess the validity of the method, authentic samples of the drug with different concentrations covering the cited concentration range were analyzed. The results obtained adopting both the spectrophotometric and the fluorimetric methods were in good agreement with those obtained using the published methods as revealed by the statistical analysis of data applying the

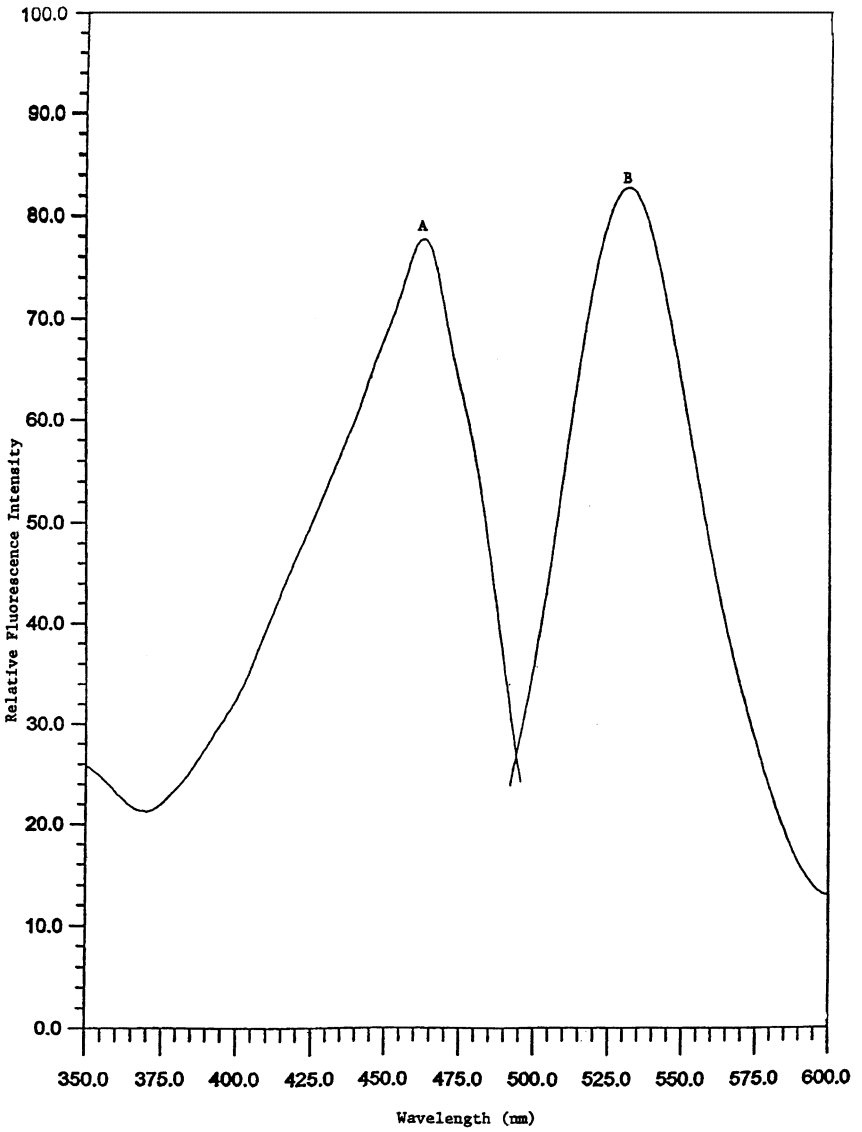


Fig. 2. Fluorescence scan for (4.8×10^{-10}) mol/ml ramipril after reaction with NBD-F (A = excitation, B = emission) using methanol as solvent.

Student's *t*-test and variance *F*-test. It is evident that there is no significant difference in the performance of the two methods regarding accuracy and precision (Table 1).

The proposed spectrophotometric method was then applied to the determination of ramipril in its dosage form (tablet). Table 2 shows that the results are in accordance with those obtained by the reference method. No interference was encountered from the common tablet excipients, such as talc, starch, lactose and magnesium stearate.

The high sensitivity obtained by the proposed fluorimetric method allowed the determination of the drug in plasma. The peak plasma level of the drug following therapeutic doses of 5–10 mg daily is 10–30 ng/ml [1,4,18]. Plasma concentration of ramipril is of clinical and pharmacological interest in cases of acute and chronic toxicity of the drug, as well as in adjusting the

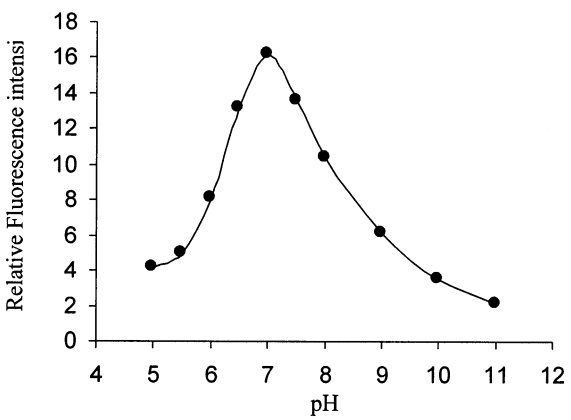


Fig. 3. Effect of different pH values on the relative fluorescence of ramipril (9.6×10^{-11}) mol/ml after reaction with NBD-F.

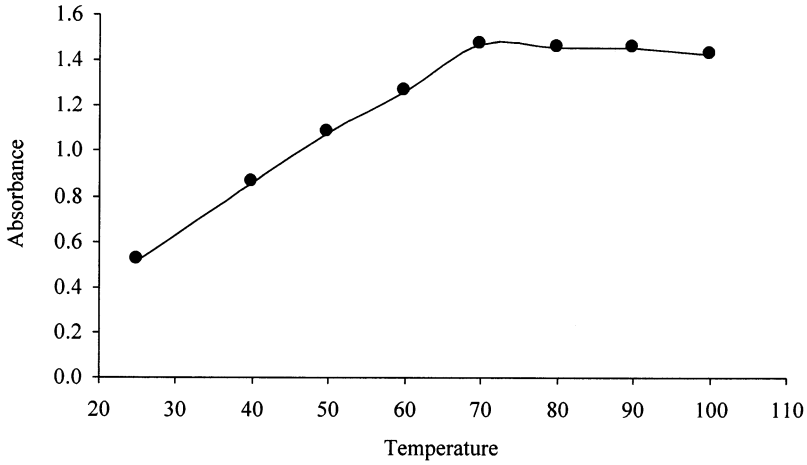


Fig. 4. Effect of temperature on the development of the reaction product of ramipril (2.16×10^{-8}) mol/ml with NBD-F at $\lambda_{max} = 465$ nm.

Table 1
Application of the proposed method to the determination of ramipril in its pure form

Spectrophotometric method			Reference method [6]	Spectrofluorimetric method			Reference method [11]
Taken (μg)	Found (μg)	Recovery (%)	Recovery (%)	Taken (ng)	Found (ng)	Recovery (%)	Recovery (%)
1	0.979	97.95	98.50	20	19.50	97.56	98.3
2	2.006	100.30	99.30	40	39.02	97.57	98.2
3	3.018	100.60	97.10	60	59.20	98.78	97.6
4	4.13	103.30	97.60	80	80.75	100.90	98.1
5	4.957	99.15	100.10	100	99.75	99.75	100.3
6	5.8	96.63	99.3				
7	6.84	97.75	96.40				
8	8.144	101.80	100.20				
9	9.054	100.60	97.90				
10	9.89	98.90	96.80				
$\bar{X}$		99.69	98.32			98.912	99.50
SD		±1.34	±1.16			±1.130	±0.75
		$F = 2.21$ *	(3.14) **			$F = 2.13$ *	(6.39) **
		$t = 1.75$ *	(2.202) **			$t = 0.53$ *	(2.776) **

* Calculated values of t- and F-test.
** Tabulated values of t- and F-test at 95% confidence limit.

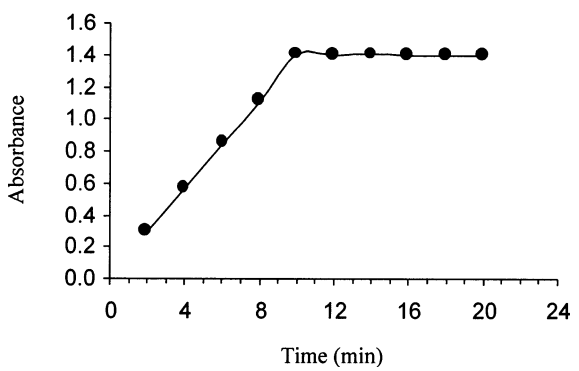


Fig. 5. Effect of the reaction time on the formation of the reaction product of ramipril (10 μg/ml) with NBD-F reagent at 70°C.

dose for patients with liver dysfunction [19,20]. Thus the proposed method was further applied to the determination of the drug in spiked human plasma. The results were accurate and precise and proved to be satisfactorily for the kinetic studies and routine estimation of the drug in human plasma.

Possible interference from hydrocholrothiazide likely to be co-administrated with ramipril was also investigated. The interference can be avoided by aqueous extraction of the drug. The interference was ±2.5% in concentration range 1–10 μg/ml.

In conclusion, NBD-F is a suitable reagent for the determination of ramipril in its dosage forms and in plasma. The suggested method is sensitive, simple, reproducible, and can be used for routine analysis in control laboratories.

Table 2
Application of the proposed method to the determination of ramipril in tablets and in human spiked plasma. The results are the average of four determinations

Samples	Recovery (%)	
	Proposed method	Reference method
1. Tritace tablets (5 mg of ramipril in tablet)	98.66 ± 0.38, $F = 5.7$ * (9.28) **, $t = 1.8$ * (3.182) **	98.76 ± 0.88
2. Human spiked plasma	99.08 ± 1.11, $F = 1.53$ * (9.55) **, $t = 1.26$ * (3.182) **	98.83 ± 1.443

* Calculated values of t- and F-test.
** Tabulated values of t- and F-test at 95% confidence limit.

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