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NOVEL 96–MICROWELL SPECTROPHOTOMETRIC ASSAYS WITH HIGH THROUGHPUT FOR DETERMINATION OF IRBESARTAN IN ITS TABLETS

I.A. DARWISH*, T.A. WANI, N.Y. KHALIL, A.H. BAKHEIT
Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia.

Two novel 96-microwell–based spectrophotometric assays have been developed and validated for determination of irbesartan (IRB) in its tablets. The two methods were based on the formation of colored charge–transfer (CT) complexes between IRB as a n–electron donor and each of 2,3–dichloro–5,6–dicyano–1,4–benzoquinone (DDQ) and p–chloranilic acid (pCA) as π–electron acceptors. The proposed assays were carried out in 96-microwell plates, and the absorbances were measured by microwell–plate reader. Under the established optimum conditions, Beer`s law was obeyed in IRB concentration ranges of 1–160 and 0.5–180 μg/ml, for DDQ and pCA methods, respectively. No interference was observed from the additives that are present in the tablets or from hydrochlorothiazide that is co–formulated with IRB in some tablets. The assays were successfully applied to the determination of IRB in tablets with good accuracy and precision. The two assays have high throughput properties, consume minimum volumes of organic solvents thus reduce the exposures of the analysts to their toxic effects, and they reduce the analysis cost by 50–fold. Although the proposed assays were validated for IRB, however, the same methodology could be used for any electron–donating analyte for which a CT reaction can be performed.

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1. Introduction

Irbesartan (IRB); (2-butyl-3-({4-[2-(2H-1,2,3,4-tetrazol-5-yl)phenyl]phenyl}methyl)-1,3-diazaspiro[4.4]non-1-en-4-one (Fig. 1), is a new member of non-peptide angiotensin II receptor antagonists used worldwide in the treatment of hypertension. IRB exerts its action mainly via a selective blockade action on AT1 receptors and the consequent reduced pressor effect of angiotensin II [1,2]. IRB may be used alone or in combination with other antihypertensive agents such as hydrochlorothiazide (HCT).

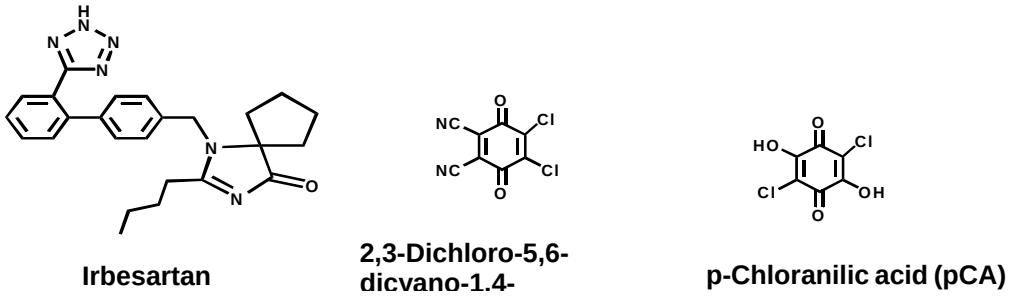


Fig. 1. The chemical structures of irbesartan and the involved π-electron acceptors

A literature survey revealed that several analytical methods were reported for determination of IRB. These methods included high-performance thin-layer chromatography [3],

*Corresponding author: idarwish@ksu.edu.sa

liquid chromatography [4–6], and adsorptive stripping voltammetry [7]. These methods were not simple to perform, time-consuming, and utilize expensive instruments that are not available in most quality control laboratories. In general, spectrophotometry is the most widely used technique in pharmaceutical analysis because of its inherent simplicity and wide availability in most quality control laboratories [8]. However, few spectrophotometric methods have been reported for the analysis of IRB in its pharmaceutical tablets [9–12]. Unfortunately, these methods suffered from major drawbacks such as employment of tedious liquid–liquid extraction procedures using large volumes of organic solvents in the methods based on formation of ion–pair associates [10], and time–consuming such as the kinetic-based methods [12]. Therefore, the development of new alternative spectrophotometric methods for determination of IRB in its pharmaceutical tablets was very essential.

The molecular interactions between the electron-donating pharmaceutical compounds and electron-accepting reagents are generally associated with the formation of intensely colored CT complexes, which usually absorb radiations in the visible region. The rapid formation of these complexes leads to their widespread utility in the development of visible spectrophotometric methods for analysis of many pharmaceutical compounds [13–17]. Literature survey revealed that the CT reaction of IRB has not been investigated yet. This fact promoted our interest in employment of the CT–reaction as a basis for the development of new spectrophotometric methods for determination of IRB. However, the conventional CT–based spectrophotometric methods that have been reported so far are not automated and consequently their throughput is low, thus their applications in pharmaceutical quality control laboratories are limited. Moreover, these methods suffer from the consumption of large volumes of organic solvents, which leads to high analysis cost, and more importantly, the incidence of exposure of the analysts to the toxic effects of the organic solvents [18–22].

For these reasons, the present study was devoted to investigate the CT reaction of IRB, and its employment in the development of novel non–conventional spectrophotometric assays with high analysis throughput and can reduce the consumption of organic solvents in the determination of IRB in tablets.

2. Experimental

Apparatus

Microwell–plate absorbance reader (ELx808, Bio-Tek Instruments Inc. Winooski, USA) was used for all the measurements in 96-microwell plates. UV-1601 PC (Shimadzu, Kyoto, Japan) ultraviolet–visible spectrophotometer with matched 1 cm quartz cells was used for recording the absorption spectra. 96-Microwell plates were a product of Corning/Costar Inc. (Cambridge, USA). Finnpipette adjustable 8–channel pipette was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Chemicals and tablets

IRB was obtained from AK Scientific Inc. (California, USA). HCT was obtained from Sigma Chemical Co. (St. Louis, USA). DDQ (Merck, Germany) was 2% (w/v) in methanol and it was prepared fresh daily. pCA (BDH Chemicals, Poole, UK) was 0.5% (w/v) in acetonitrile and it was prepared fresh daily. Approvel® tablets (Sanofi Winthrop Industrie, Carbon Blanc Cedex, France) labeled to contain 300 mg IRB were obtained from the local market.

Preparation of stock standard and tablet sample solutions

Into a 5–ml calibrated flask, 10 mg of IRB was accurately weighed, and dissolved in 10 ml methanol or acetonitrile for DDQ– and pCA–based methods, respectively. These stock solutions were diluted with the corresponding solvents to obtain IRB concentrations in the range of 1–160 and 0.5–180 μg/ml, for DDQ– and pCA–based methods, respectively. For tablet solutions, twenty tablets were weighed and finely powdered. A quantity of the powder equivalent to 20 mg of IRB was transferred into a 10–ml calibrated flask, dissolved in 4 ml methanol for reaction with DDQ or in acetonitrile for reaction with pCA, swirled and sonicated for 5 min, completed to volume with the corresponding solvent, shaken well for 15 min, and filtered. The first portion of

the filtrate was rejected, and a measured volume of the filtrate was diluted quantitatively with the corresponding solvent to yield the suitable concentrations that lie in the linear range of each assay.

General analytical procedure

Accurately measured aliquots (100 µl) of the standard or sample solution containing varying amounts of IRB (1–160 and 0.5–180 µg/ml, for DDQ and pCA, respectively) were transferred into wells of 96-microwell assay plates. One hundred microliters of DDQ solution (2%, w/v) or pCA solution (0.5%, w/v) was added, and the reaction was allowed to proceed at room temperature (25 ± 1 °C) for 5 min. The absorbances of the resulting solutions were measured by the microwell–plate reader at 460 and 490 nm for reactions with DDQ and pCA, respectively. Blank wells were treated similarly except 100 µl of the solvent was used instead of sample, and the absorbances of the blank wells were subtracted from those of the other wells.

Determination of molar ratio

The Job's method of continuous variation [23] was employed. Master equimolar solutions (2×10^{-3} M) of IRB and each of DDQ and pCA were prepared. Series of 200 µl portions of the master solutions of IRB with acceptor were made up comprising different complementary ratios (0:10, 1:9, ……, 9:1, 10:0, inclusive) in each well of the 96-microwell assay plate. The reaction was allowed to proceed at room temperature (25 ± 1 °C) for 5 min. The absorbances of the developed colors were measured by the microwell plate reader at 460 and 490 nm for DDQ– and pCA–based methods, respectively. Blank wells treated similarly except solvent was used instead of IRB sample. The measured absorbances were plotted as a function of IRB mole fraction. The generated plots were used for determination the molar ration of IRB with each of DDQ and pCA.

Molecular modeling for the CT complexes of IRB with DDQ and pCA

The molecular modeling for the CT complexes was performed by using CS Chem3D Ultra, version 9 (Cambridge Soft Corporation, Cambridge, MA, USA) implemented with molecular orbital computations software (MOPAC), and molecular dynamics computations software (MM2).

3. Results and discussion

Strategy for assay development and its design

In the present study, IRB was selected based on its therapeutic importance, clinical success, and the expected electron–donating ability. Previous studies involving CT reactions with polyhalo–/polycyanoquinone electron *n*–acceptors revealed that DDQ and pCA are the most efficient reagents in terms of their reactivity [15,24]. Furthermore, their CT reactions with electron-donating analytes are instantaneous [15,16]. For these reasons, DDQ and pCA were used as electron acceptors in the development of the proposed assays. The proposed assays were designed to employ 96–microwell assay plate as the CT reactions were carried out in microwells of the assay plate (200–µl reaction volume). The solutions were dispensed by 8–channel pipette, and the absorbances of the colored CT complexes were measured by 96–microwell plate absorbance reader. The 96–microwell design of the proposed assay was based on the previous success of Darwish *et al.* [25] in the utility of this design for determination of some other pharmaceuticals.

Reaction and spectral characteristics

The interactions of IRB with DDQ and pCA were allowed to proceed at room temperature, and the absorption spectra of the produced chromogens were recorded. IRB gave red chromogens showing absorption maxima at 460 and 520 nm with DDQ and pCA, respectively. These bands were attributed to the formation of the radical anions (DDQ^{-} and pCA^{-}) [26], which were probably formed by the dissociation of the original donor-acceptors (D-A) complexes:

$$D + A \rightleftharpoons \underset{\text{complex}}{(D\text{-}A)} \overset{\text{polar solvent}}{\rightleftharpoons} \underset{\text{radical ions}}{D^{\cdot +} + A^{\cdot -}}$$

Optimization of experimental conditions

The experimental conditions affecting the reactions in the 96–well format were optimized by altering each reaction variable in a turn while keeping the others constant. IRB–DDQ CT complex was measured at 460 nm. However, IRB–pCA complex was measured at 490 nm, although it exhibited maximum absorption peak at 520 nm. This has been done because 520 nm–filter was not available in the plate reader, and the closest available wavelength filter was 490 nm. The results of variations in the DDQ and pCA concentrations indicated that 100 µl of 2 and 0.5% (w/v) were the optimum concentration for DDQ and pCA, respectively, as these concentrations gave the highest absorbances. Previous studies [27] demonstrated that the interaction of electron-donors with DDQ and pCA in polar solvents (e.g. methanol and acetonitrile) produces CT complexes with molar absorptivity values higher than those produced in non-polar solvents (e.g. chloroform). Different polar solvents were tested as reaction solvent; these solvents were methanol, ethanol, 1–propanol, 1–butanol, and acetonitrile. Methanol and acetonitrile offered the highest sensitivity with DDQ and pCA, respectively; therefore these solvents were used for the subsequent experiments. The optimum reaction time was determined by monitoring the color development in the microwells at room temperature (25 ± 1 °C). Complete color development was attained instantaneously in both cases, however for higher precision readings, the reactions were allowed to proceed for 5 min. The developed colors remained stable at room temperature for at least a further 30 min in both cases. A summary for the optimum conditions is given in Table 1.

Table 1. Optimum conditions for the charge-transfer reaction of IRB with DDQ and pCA

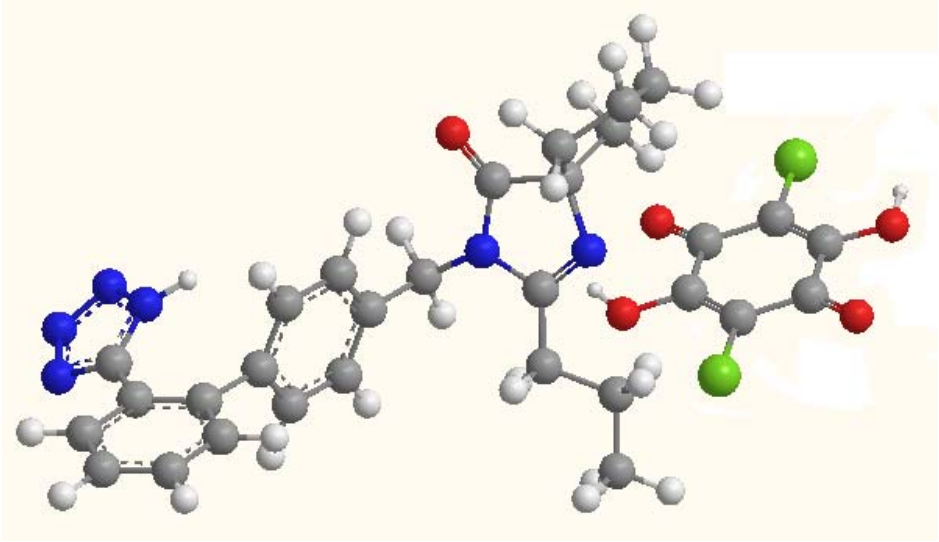
Condition	Studied range	Optimum	
		DDQ	pCA
Acceptor conc. (%, w/v)	0.1 ─ 4	2	0.5
Solvent	Different [a]	Methanol	Acetonitrile
Reaction time (min)	0 ─ 30	5	5
Temperature (°C)	25 ─ 60	25	25
λ_{max} (nm)	400 ─ 600	460	520[b]

[a] Solvents tested: methanol, ethanol, 1–propanol, 1–butanol, and acetonitrile.
[b] Measurements were carried out at 490 nm.

Molar ratio of the reaction, molecular modeling, and site of interaction

Job's method of continuous variation [23] was used for determining the molar ratio of IRB to each of DDQ and pCA. From the obtained Job's plots, it was concluded that the IRB:DDQ and IRB:pCA ratios were 1:1. This indicated that only one site of interaction was involved in the formation of the colored CT complexes in spite of the presence of more than one possible electron–donating site in IRB structure (e.g. oxygen atom of the carbonyl group, nitrogen atoms of the imidazole ring, and nitrogen atoms of the tetrazole ring). For investigating the site of interaction, modeling for the CT complexes was performed. IRB was energy-minimized alone and with each of DDQ and pCA. It was found that the electron densities in IRB molecule that are located on the oxygen atom of the carbonyl group, nitrogen atom of the imidazolone (–N–CH_2–phenyl), and nitrogen atoms of the tetrazole ring were comparable (–0.44729, –0.33642, and –0.33313, respectively). As well, it was found that DDQ and pCA moved toward the nitrogen atom of the imidazolone (–N–CH_2–phenyl) of IRB to form the CT complex. The data was presented for pCA (Fig. 2), and the same behavior was obtained with DDQ. These facts, taking the molar ratios in account, confirmed that only the nitrogen atom of the imidazolone

(–N–CH_2–phenyl) of IRB was involved in the formation of CT complexes. The other anticipated centers did not contribute in the CT reactions, probably due the steric hindrance effect of the IRB molecules [28].



IRB **pCA**

Fig. 2. Energy-minimized CT complex of IRB with pCA

Validation of the proposed assays

Linearity and sensitivity. Under the above mentioned optimum reaction conditions, the calibration curves for the determination of IRB by the proposed assays were constructed by plotting the absorbances as a function of the corresponding concentrations. The regression equations for the results were derived using the least-squares method. Beer's law plots (10-points) were linear in the ranges of 1–160 and 0.5–180 μg/ml for DDQ– and pCA–based assays, respectively. The limits of detection (LOD) and quantitation (LOQ) were determined [29] 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. The LOD and LOQ values with the other quantitative parameters of the proposed assays are given in Table 2.

Table 2. Quantitative parameters for the analysis of IRB by the proposed assays

Parameter	Value	
	DDQ	pCA
Range (μg/ml)	1–160	0.5–180
Intercept	0.0363	0.0189
Slope	0.1351	0.1141
Correlation coefficient	0.9991	0.9958
LOD (μg/ml)	0.27	0.14
LOQ (μg/ml)	0.89	0.47

Accuracy and precision. Accuracy of the proposed assays was assessed by analytical recovery studies. Recovery was determined by the standard addition method. Known amounts of IRB were added to pre–determined IRB–containing tablets, and then determined by the proposed assay. The mean analytical recovery was calculated and found to be 96.8–101.5 ± 0.8–1.6% indicating the accuracy of the proposed assays. The precisions of the proposed assays were determined on samples of IRB solutions at three concentration levels for each drug (Table 3) by

analyzing 5 replicates of each sample as a batch in a single assay run. The relative standard deviations (RSD) did not exceed 1.54% (Table 3) proving the high precision of the assays for the routine application in quality control laboratories. This high level of precision was attributed to the accuracy of the volumes that have been dispensed in the microwells by multi–channel pipettes, and completeness of the reaction in the small volume (200 µl).

Table 3. Precision of the proposed assays at different IRB concentrations

Concentration (µg/ml)	Relative standard deviation (%), n = 5			
	DDQ		pCA	
	Within–assay	Between–assays	Within–assay	Between–assays
10	0.85	1.08	0.52	1.47
80	1.02	1.54	0.88	1.45
150	1.25	1.36	1.42	1.05

Selectivity. The proposed assays have the advantages that the measurements are performed in the visible region, away from the UV-absorbing interfering substances that might be co-extracted from tablets that contain IRB. The interference from HCT that is co-formulated with IRB in some of its tablets was studied. Potential interference of HCT was studied in a ratio which is normally present in their combined tablets. No interference from HCT was found with IRB in the proposed assays. This specificity of the CT reaction for IRB was attributed to its basic character, which allows the CT, rather than HCT, that does not have sufficient basicity to achieve CT reaction (pKa = 7.9) [30]. As well, no interference was observed from the excipients with the proposed assays as indicated from the obtained good recovery (mentioned above). The absence of interference from the excipients, even though they contain basic component(s) was attributed to the extraction of the IRB tablets prior to the analysis with organic solvents (methanol or acetonitrile) in which the excipients do not dissolve.

Application of the proposed assays in the analysis of IRB tablets

The commercially available tablets of IRB (Approvel® tablets) were subjected to the analysis by the proposed and reported methods [4], and the obtained results were then statistically compared with each other. The mean percentage recoveries, relative to the labeled amounts, obtained by the proposed assays were 100.7 ± 1.76 and 101.0 ± 1.73% by DDQ and pCA assays, respectively. Values for t- and F-tests were calculated and found to be lower than the tabulated ones indicating that there were no significant differences between both the proposed and the reported assays at 95% confidence level in terms their accuracy and precision.

4. Conclusions

The present study described the development and validation of two novel microwell-based spectrophotometric assays for the determination of IRB based on its CT reaction with DDQ and pCA reagents. In these assays, the CT reactions were carried out in 96-microwell plates (200-µl reaction volume) instead of the conventional volumetric flasks (10,000-µl volume). The absorbances were measured by microwell–plate reader instead of the conventional spectrophotometer. The assays described herein offered the following advantages:

– Reduction in the consumption of organic solvents in the CT–based spectrophotometric analysis, accordingly reduction in the exposures of the analysts to the toxic effects of organic solvents.

– Reduction in the analysis cost by 50–folds which can be reflected on the price for the finished dosage forms, thus it can reduce the expenses for the medications.

– Providing a high throughput analytical methodology that can facilitate the processing of large number of samples in a relatively short time. This property was attributed to the use of multi-channel pipettes for efficient dispensing of the solutions, carrying out the analytical reaction in 96–well plates (as reaction vessels), and measuring the color signals in the

96 wells at ~ 30 seconds by the plate reader.

– Although the proposed assays were developed and validated for IRB, however, it is also anticipated that the same methodology could be used for essentially any analyte that can exhibit CT reaction.

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