Drug Development and Industrial Pharmacy, 27(1), 39–45 (2001)

RESEARCH PAPER

High-Performance Liquid Chromatography Assay for Indorenate in Pharmaceutical Powders

J. R. Villalobos-Hernandez, R. Arenas-Hernandez, and L. Villafuerte-Robles*

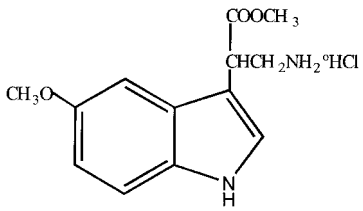
Department of Pharmacy, National School of Biological Sciences, National Polytechnic Institute, D. F. Mexico

ABSTRACT

A high-performance liquid chromatographic (HPLC) method for quantification of indorenate admixed of pharmaceutical excipients (Pharmatose DCL 21, Povidone USP and Helmcel 200) is described. Indorenate was extracted from the mixtures using a mobile phase composed of acetonitrile and a sodium acetate buffer solution 0.1 M (63:37) and separated from other dissolved components by ion supression-HPLC. The method was standardized using a C-18 column (250 mm × 4.8 mm, i.d., 5 μm). The photometric detector was fixed at 228 or 272 nm depending on the admixed excipients. Validation parameters included linearity, precision, accuracy, reproducibility, and specificity. The method was specific, selective, and capable to distinguish indorenate from their degradation products and the antihypertensive pelanserine.

INTRODUCTION

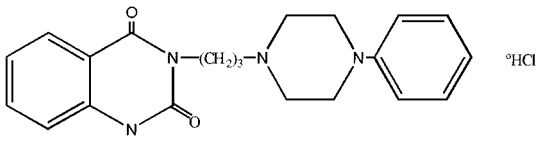
Indorenate, chemically named 5-methoxytryptamine, β-methyl carboxylate hydrochloride, is an experimental drug designed as a 5-HT_1 agonist (1–3).



*To whom correspondence should be addressed. Carpio y Plan de Ayala s/n, C.P. 11340, D. F. Mexico. Fax: (52) 5396 3503.

This compound is known to possess antihypertensive and antianxiety properties. When administered orally, this drug has been proved to be well absorbed, rapidly metabolized, and excreted (4,5). To assay indorenate in biological fluids, a high-performance liquid chromatography (HPLC) method coupled to an electrochemical detector has been used (6). However, the actual development of this experimental drug in a pharmaceutical dosage form makes necessary an analytical methodology capable to determine indorenate in mixtures with some excipients and to detect the molecule in the presence of possible degradation products. The development of this method includes the evaluation of parameters like linearity of the system and method, precision, accuracy, repeatability, and specificity (7,8). This process allows the design of a reliable, accurate, and interpretable set of information describing the method (9,10). Moreover, considering that stability is an integral part of pharmaceutical development, the stability-indicating ability of the method should be also determined (11). This stability-indicating ability is most often tested by subjecting a drug solution to stress conditions. The stress is applied by changing the pH of the solution to extremes and heating (12), although agents causing oxidation can also be used with the same purpose.

The development of the method is based in HPLC coupled to an UV detector because other related products like pelanserine have been successfully assayed with this methodology (13).



This paper describes a method to analyze indorenate admixed of pharmaceutical excipients and in the presence of degradation products obtained by effect of pH and hydrogen peroxide.

MATERIALS AND METHODS

Reagents and Chemicals

The experimental drugs indorenate and pelanserine were obtained from CINVESTAV-IPN, Mexico. HPLC grade acetonitrile and water and the analytical reagent-grade sodium acetate, glacial acetic acid, hydrochloric acid, and hydrogen peroxide were purchased from J.T. Baker, Mexico. Microcrystalline cellulose (Helmcel 200), polyvinylpyrrolidone (Povidone USP), and lactose (Pharmatose DCL 21) were obtained from Helm, Mexico.

HPLC Instrumentation and Conditions

An HPLC system (Beckman Inc) consisting of a solvent delivery system model 128 was used. A Rheodyne injector model 7525i fitted with a 20-μL loop, a multiple UV-VIS wave length detector (Beckman model 168), and a printer (Epson model 870) was used. The column was a Phenomenex C_{18} 250 mm $\times$ 4.6-mm i.d., with 5-μm particles.

The flow rate of the mobile phase was set at a flow rate of 1 mL/min and maintained during the analysis. The mobile phase was a mixture of acetonitrile and a sodium acetate pH 4.00 buffer solution 0.1 M (63:37). The mobile phase was filtered through a 0.22-μm filter to remove any particulate matter and degassed sonically during 10 minutes before use. The UV detector was set either at 228 or 272 nm depending on the excipient admixed. The elution was carried out under isocratic conditions at room temperature

Linearity and Precision of the System

For the preparation of a standard stock solution of indorenate, 26.9 mg of the drug was weighed, transferred into a 50-mL volumetric flask, and diluted with mobile phase to give a concentration of 538 μg/mL. This standard was further diluted to set calibration curves. The same procedure was used to prepare a pelanserine standard solution. This was used to verify the capability of the selected HPLC conditions to separate indorenate from other substances. Subsequently pelanserine was used as a marker to check the suitability of the system. A sample of 20 μL of a dilution containing equal quantities of indorenate and pelanserine (107.6 μg/mL) was injected.

Two series of standard solutions of 1.08, 5.38, 10.76, 26.90, 53.80, 107.60, and 161.40 μg/mL of indorenate diluted in mobile phase were prepared from the standard stock solution. Twenty microliters from each dilution were injected into the chromatograph and analyzed in duplicate. The peak areas for indorenate were recorded for all the chromatograms. A calibration curve was constructed by plotting peak areas against the concentration of the drug in the final solution. A linear regression analysis of data obtained for the first series at the wave length of 228 nm and for the second series at 272 nm was calculated. The peak areas for indorenate standard were linearly related to indorenate concentration over the range of 1 to 161 μg/mL. The equations obtained by linear regression were used to calculate the indorenate concentration in the samples containing excipients.

The precision of the assay was determined by injecting twice two series, each one from a different standard

solution of indorenate containing 107.6 μg/mL six times, each time calibrating the detector at a wave length of 228 or 272 nm. The average response, standard deviation, variation coefficient, and confidence limits were calculated.

Linearity, Precision, and Accuracy of the Method

To evaluate possible interactions with excipients, in a first part, 2.69 g of Helmcel 200, 53.8 mg of PVP, and 26.9 mg of indorenate were dispersed on 50 ml of mobile phase, applying magnetic stirring during 15 min. This suspension was then filtered through 0.22-μm membrane filter. The same procedure was used to prepare a second standard solution with excipients, substituting Helmcel 200 with Pharmatose DCL 21, using the same proportions. Both types of standards were further diluted to set calibration curves.

Two series of standard solutions containing indorenate at concentrations of 1.08, 10.76, 26.90, 53.80, 107.60, and 161.40 μg/mL obtained from standard solutions added of excipients and diluted in mobile phase were prepared. Twenty microliters from each dilution was injected and analyzed in duplicate. The detection of indorenate was made at 228 nm in the case of samples containing lactose and at 272 nm for samples containing cellulose. The estimated quantity of indorenate versus the expected quantity of itself (in micrograms) was performed. The calculation of statistic parameters was accomplished.

The precision of the method was determined using the following procedure. Two analysts injected twice 20 μL of each of three standard solutions containing 107.6 μg/mL of indorenate and the corresponding added excipients on two different days. Two series were made setting each one to a given wave length, 228 or 272 nm. The variation coefficient was calculated, and the sources of variation of the method were set by ANOVA. A nested design was supposed for the model.

The estimation of the method accuracy was made with the following procedure. Twelve standards added of excipients and containing 107.6 μg/mL of the drug were chromatographed in duplicate, and the average response, standard deviation, and confidence limits were calculated. Two series were made setting each one to a given wave length, 228 or 272 nm.

Stability Indicating Nature of the Assay

The drug was subjected to extreme acidic, alkaline, and oxidative conditions to promote possible degradations. A standard solution containing 538 μg/mL of indorenate was prepared. Three samples of 10 ml of this standard were added to 2.7 mL of the following solutions (1) concentrated HCl diluted with water (3:7 v/v), (2) NaOH 30% w/v, and (3) H_2O_2 (30%) to promote degradation of the drug. The acid and alkaline solutions were allowed to stand for a few minutes and adjusted to a final volume of 50 mL with mobile phase. In the case of the oxidative conditions, the solution was allowed to stand for 2 hr before the adjustment to the final volume of 50 mL with mobile phase was made. All the samples were chromatographed.

RESULTS AND DISCUSSION

When the solution containing indorenate and pelanserine was injected onto the chromatographic column, a clear separation of these compounds occurred, as seen in Figure 1. The retention times for indorenate and pelanserine were 2.8 and 4.0 min, respectively.

The calibration plot providing the peak areas on the y-axis against concentration of indorenate on the x-axis showed the linearity of the system. This was excellent according to a determination coefficient of 1.000, when the detection was made at 228 nm. When the detection was performed at 272 nm, the determination coefficient was 0.9999, as seen in Figure 2.

Data for the precision of the system are given in Table 1. The percent relative standard deviation determined from six assays of an indorenate standard solution was found to be 0.150% when the detection was performed at a wave length of 228 nm. By detection at 272 nm, the relative standard deviation was 0.406%.

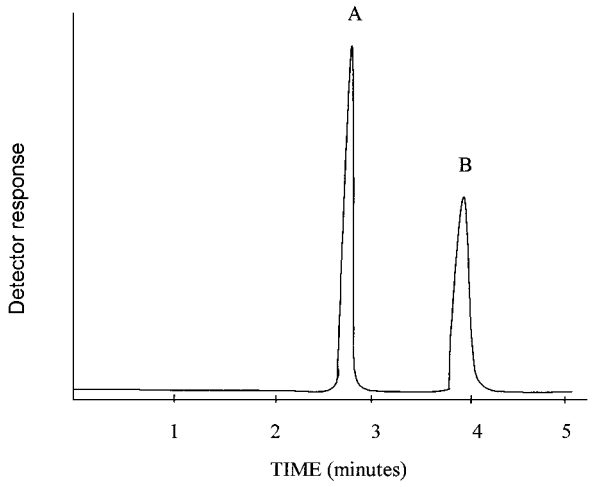


Figure 1. Chromatogram showing separation of indorenate (A) from the pelanserine (B).

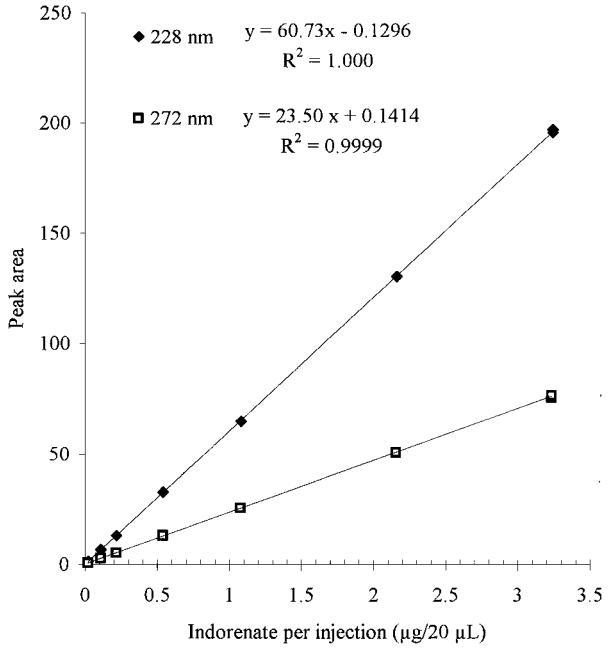


Figure 2. Calibration plot for the indorenate HPLC assay with UV detection at 228 and 272 nm. Each regression calculated with 28 points.

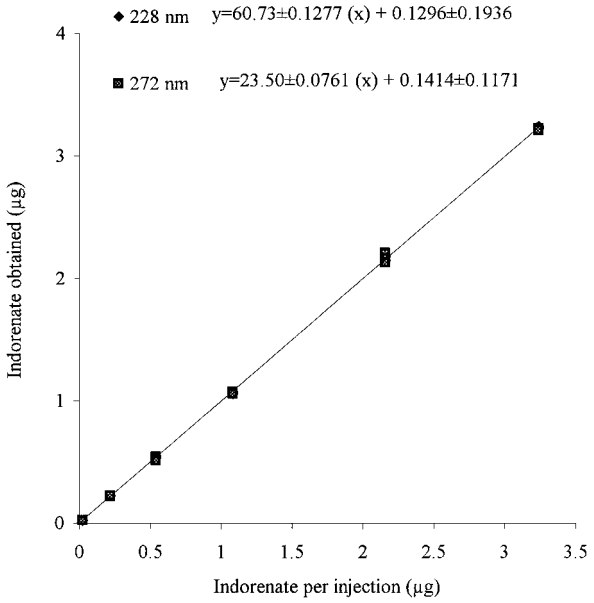


Figure 3. Method linearity for HPLC assay of indorenate. Regression calculated with 24 individual points.

Concerning the method of analysis, the relationship between the estimated quantity of indorenate obtained from samples loaded with excipients and the theoretical or expected quantity in the sample was linear. This relationship not only maintained the linearity but also showed no interference of the excipients. This could be inferred from determination coefficients and slopes with values close to 1 and constants close to 0, as seen in Figure 3.

Table 1

Precision of the System for the Indorenate HPLC Assay

Assay (228 nm)	Recovery (%)	Assay (272 nm)	Recovery (%)
1	99.73	1	99.70
2	99.83	2	99.73
3	100.09	3	100.55
4	100.11	4	100.55
5	100.00	5	100.44
6	100.02	6	100.44
Average	99.96		100.23
SD	0.1503		0.4067
Relative SD	0.1504		0.4058
95% CL	99.96 ± 0.1578		100.23 ± 0.4270

The precision of the method was estimated through intraday, interday, and interanalyst reproducibility analysis. The intraday reproducibility was determined by two analysts through replicate injections of standard solutions loaded with excipients and prepared before analysis. The interday reproducibility of the method was determined by six repeated analysis of excipients loaded standard solutions, three per analyst, on two different days. The results are shown in Table 2. ANOVA was performed with a

Table 2

Precision for Analyst and Day of Analysis for the Indorenate HPLC Assay with UV-Detection at 228 and 272 nm

	Analyst, 228 nm		Analyst, 272 nm	
Day	1	2	1	2
1	100.90	99.42	106.75	105.46
	101.07	98.84	105.75	105.06
	100.68	99.21	105.04	103.59
2	100.11	98.96	104.16	104.24
	99.72	99.87	104.78	104.39
	100.92	99.63	104.89	103.37

$SD_{228\,nm} = 0.7917$.
$SD_{272\,nm} = 0.9382$.
Relative $SD_{228\,nm} = 0.7922$.
Relative $SD_{228\,nm} = 0.8953$.

Table 3

Precision of the Method of Indorenate Assay: ANOVA to Estimate the Reproducibility by Different Analysts and on Different Days

Source	Square Sum	Degrees of Freedom	Mean Square	F	$F_{(0.05)}$ Crit.
(228 nm)					
Analyst	4.6905	1	4.6905	12.20	18.51
Day (analyst)	0.7689	2	0.3844	2.14	4.46
Error	1.4369	8	0.1796		
Total	6.8963	11			
(272 nm)					
Analyst	2.3275	1	2.33	1.53	18.51
Day (analyst)	0.7689	2	1.52	2.81	4.46
Error	4.3218	8	0.54		
Total	9.6833	11			

$F_A < F_{A\,crit.\text{-}0.05}$.
$F_D < F_{D\,crit.\text{-}0.05}$.

confidence interval of 95% to compare the difference of the mean recoveries. The results in Table 3 show that no significant difference could be attributed to either different analysts or different days.

The accuracy of the method was obtained from the recovery data (Table 4). The average recoveries were 99.61 and 100.82% when using respectively 228 and 272 nm as wave lengths for the indorenate detection. Following the same order, the confidence limits were ±0.5138 and ±0.833%.

Table 4

Accuracy of the Method for the Indorenate HPLC Assay

Assay (228 nm)	Recovery (%)	Assay (272 nm)	Recovery (%)
1	100.73	1	100.77
2	101.03	2	100.86
3	98.74	3	100.86
4	98.09	4	100.74
5	99.47	5	102.43
6	99.21	6	102.24
7	99.78	7	102.19
8	100.23	8	102.33
9	99.54	9	99.94
10	99.87	10	99.99
11	99.39	11	98.79
12	99.28	12	98.7
Average	99.61		100.82
SD	0.8085		1.311
Relative SD	0.8117		1.30
95% CL	99.614 ± 0.514		100.82 ± 0.833

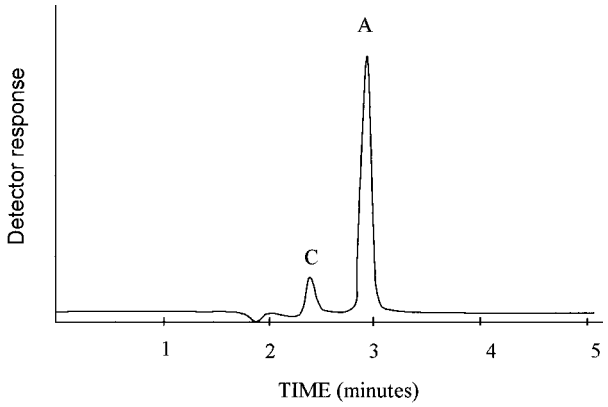


Figure 4. Chromatogram for the indorenate sample (A) stressed under acid conditions (HCl) showing a small peak for a degradation product (C).

The stability-indicating nature of the HPLC assay was determined by subjecting indorenate to extreme acidic, basic, and oxidative conditions. The indorenate solution stressed under acidic conditions showed a peak for a degradation product at 2.35 min, as seen in Figure 4. This chromatogram shows a complete separation between the degradation product and indorenate. No interference from the degradation peak with indorenate is expected because the retention time of the drug is more than that for the degradation product. The indorenate solution stressed under alkaline conditions showed a peak for the drug and

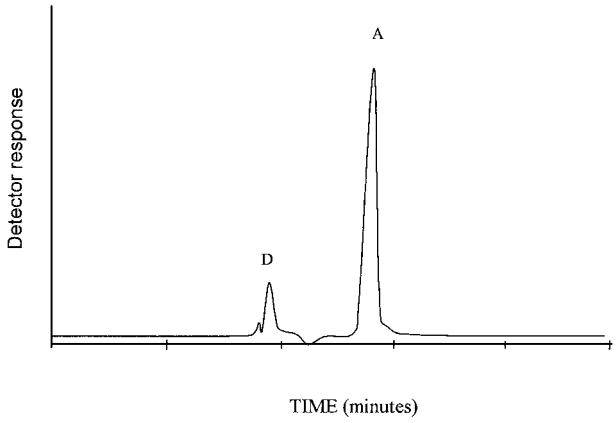


Figure 5. Chromatogram for the indorenate sample (A) stressed under basic conditions (NaOH) showing a small peak for a degradation product (D).

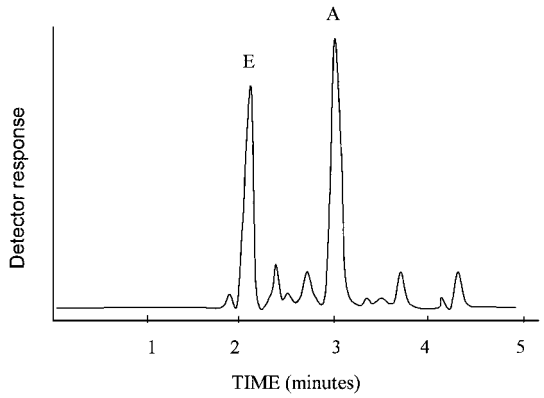


Figure 6. Chromatogram for the indorrenate sample (A) stressed under an oxidative condition (H_2O_2) showing a main peak (E) and some other small peaks for degradation products.

a peak at 2.02 min. As seen in Figure 5. The retention time for the degradation product is less than that obtained under acidic conditions. Both the peaks obtained under alkaline and acidic conditions corresponding to degradation products and the drug showed complete separation. The indorenate solution stressed under oxidative conditions (H_2O_2) showed a main peak at 2.03 min and some small peaks for degradations products, as seen in Figure 6. All peaks showed complete separation. The above results demonstrate that the HPLC assay procedure is stability-indicating.

CONCLUSION

The investigation has developed stability indicating HPLC procedure for the quantification of indorenate associated to excipients commonly used in solid dosage forms. The calibration plot obtained using this method of analysis resulted in a determination coefficient of 1.000. This linearity of the system was maintained in the method, and no interference of the excipients was observed. ANOVA performed to estimate the reproducibility of the method showed no significant differences in samples analyzed by different analysts and on different days, within an interval of confidence of 95%. The excellent accuracy of the method obtained from recovery data was 99.61 and 100.19% when the indorenate detection was made at the wave lengths of 228 and 272 nm, respectively. The analytical method has been shown to be stability-indicating through the analysis of samples stressed under acidic, alkaline, and oxidative conditions. The results have shown that there is no interference from any of the degradation products.

The purposed method was linear, accurate, precise, reproducible, and sensitive enough for the determination of indorenate and indorenate in the presence of common pharmaceutical excipients such as Helmcel 200, Pharmatose DCL 21, and polyvynilpyrrolidone. Moreover, because this method was capable of separating indorenate from degradation products, it can be applied in either further stability studies or quality-control assays.

REFERENCES

1. Hong, E.; Rión, R.; Nava-Felix, P. Characterization of the Receptors Involved on the Central Antihipertensive Effect of TR 3369. Fed. Proc. **1980**, *39*, 963.
2. Benitez-King, G.; Chavez, J.L.; Martinez, J.; Benitez-King, G.; Antón-Tay, F.; Hong, E. Further Evidence that Indorenate is a 5-HT1 Agonist. Proc. West. Pharmacol. Soc. **1991**, *34*, 433–437.
3. Hong, E.; Villalón, C.M. External Carotid vasodilatation induced by serotonin and indorenate. Proc. West. Pharmacol. Soc. **1998**, *31*, 99–101.
4. Fernández-Guasti, A.; Hong, E.; López-Ruvalcava, C. Species Differences in the Mechanism Through Which the Serotonergic Agonist Indorenate and Ipsapirone Produce Their Anxiolitic Action. Psychopharmacology **1992**, *107*, 61–68.
5. Benitez-King, G.; Antón-Tay, F.; Hong, E. Characterization of Indorenate Effects on Brain Monoamine Metabolism. Drug G. Dev. Res. **1991**, *23*, 325–331.
6. Hong, E.; Rión, R.; Aceves, J.; Benitez-King, G.; Antón-Tay, F. Further Evidence for a Central Antyhypertensive Effect of Indorenate. Proc. West. Pharmacol. Soc. **1987**, *30*, 1–3.
7. Ivanovic, D.; Medenica, M.; Radulovic, D.; Djugumovic, Z. Pharmaceutical Analysis of β-Methyldigoxin in Dosage Forms Using RP-HPLC. Drug. Dev. Ind. Pharm. **1995**, *21* (15), 1789–1797.
8. Haribhakti, R.; Alexander, K.; Parker, G. An Improved High Performance Liquid Chromatography Assay for Acetazolamide. Drug. Dev. Ind. Pharm. **1991**, *17* (6), 805–815.
9. Wieling, J.; Hendricks, G.; Tamminga, W.J.; Hempenius, J.; Mensink, C.K.; Oosterhuis, B.; Jonkman, J.H.G. Rational Experimental Design for Bioanalytical Methods Validation. Illustration Using an Assay Method for Total Captopril in Plasma. J. Chromatogr. A. **1996**, *730*, 381–394.
10. Jenke, D. Chromatographic Method Validation: A Review of Practices and Procedures. I. General Concepts and Guidelines. J. Liq. Chrom. Rel. Technol. **1996**, *19* (5), 737–757.
11. Argekar, A.P.; Sawant, J.G. Simultaneous Determination of Pyridoxine Hydrochloride and Doxilamine Succinate From Tablets by Ion Pair Reversed-Phase High-Performance

Liquid Chromatography (RP-HPLC). Drug Dev. Ind. Pharm. **1999**, *25* (8), 945–950.
12. Alexander, K.S.; Sundar, S.; Vengala, K.S.; Dollimorer, D. An improved High-Performance Liquid Chromatography Assay for Spironolactone Analysis. Drug. Dev. Ind. Pharm. **1998**, *24* (2), 101–2107.
13. Flores, M.F. Estudios Farmacocinéticos de la pelanserina. Ph.D. Thesis. CINVESTAV-IPN, 1991.