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Short communication

Sensitive high-performance liquid chromatographic method for the determination of methyl N-[5-[[4-(2-pyridinyl)-1-piperazinyl]carbonyl]-1*H*-benzimidazol-2-yl] carbamate in rat blood☆

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Abstract

A sensitive high-performance liquid chromatographic assay has been developed and validated for the determination of methyl N-[5-[[4-(2-pyridinyl)-1-piperazinyl]carbonyl]-1*H*-benzimidazol-2-yl] carbamate (CDRI compound 81/470) in normal rat blood. The method described herein is simple, with improved selectivity and sensitivity over a previously reported HPLC method. The limit of quantitation is 10 ng/ml (method 1) and 2.5 ng/ml (method 2) in blood, as compared with 40 ng/ml for the previous method. The standard curve in blood is linear over the concentration range 10–1000 ng/ml in method 1 and 2.5–1000 ng/ml in method 2 and the extraction recovery is higher than 80% for both methods.

1. Introduction

Methyl N-[5[[4-(2-pyridinyl)-1-piperazinyl]-carbonyl]-1-H-benzimidazol-2-yl] carbamate (CDRI compound 81/470; I, Fig. 1) is a new broad spectrum anthelmintic agent [1,2]. The compound possesses efficacy against developing and adult helminths by oral and parenteral routes [3]. The remarkable efficacy of I by dermal application suggests that the compound is absorbed from skin to act on both tissue dwelling and intestinal parasites [4]. The compound has also been found to possess chemoprophylactic action against some experimental nematode infections [5]. The broad spectrum anthelmintic efficacy of I, coupled with a high therapeutic index [2] lends strong support to its development as an ideal anthelmintic drug for veterinary and medical use and allows for population based chemotherapy of helminthiasis.

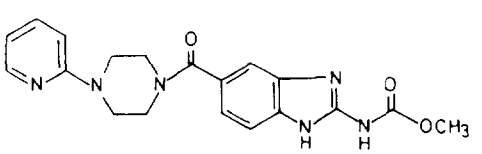

Fig. 1. Structure of C.D.R.I. Compound 81/470 (I).

A high-performance liquid chromatographic (HPLC) method for the estimation of I in normal rat serum and its application to study the

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pharmacokinetics in rats after a single bolus dose of 5 mg/kg by i.p. route has been reported [6]. The method involves precipitation of serum proteins with acetonitrile and injecting the supernatent directly onto the HPLC system. In the reported method, 0.2 ml of rat serum was used for the analysis and hence during pharmacokinetic study, five rats were sacrificed at each time point to collect serum. The limit of quantitation (LOQ) was 40 ng/ml with fluorescence detection. The lack of sensitivity, the possible high inter-subject variations because of different rats at each time point and the high cost of the study in routine pharmacokinetic and bioavailability studies of I necessitated the development of an alternative and sensitive HPLC method for the analysis of I.

In this paper, we describe a sensitive HPLC assay method for analysis of I in rat blood, of which method 1 involves precipitation of endogenous proteins by acetonitrile (LOQ = 10 ng/ml) and method 2 involves extraction of I with diethyl ether (LOQ = 2.5 ng/ml).

2. Experimental

2.1. Chemicals and reagents

Pure reference standard of I was obtained from the Pharmaceutics Division of this institute. HPLC grade chloroform and methanol were procured from E.Merck (Bombay, India), and acetonitrile from Spectrochem (Bombay, India), and used without further purification. Diethyl ether (anaesthetic grade IP, Ether India, Bombay, India) was purified before use, by washing with potassium hydroxide followed by distillation. All other reagents were of analytical grade and used without further purification.

2.2. Apparatus and chromatographic conditions

The chromatographic conditions for both methods were identical. The apparatus used for this work consisted of a HPLC pump (Kontron, Model 600, Zurich, Switzerland), coupled with a fluorescence HPLC monitor (Shimadzu, Model RF-530, Kyoto, Japan), set at excitation and emission wavelengths of 295 and 375 nm, respectively. Samples were introduced through a fixed 50-μl loop injector (Rheodyne, Model 7125, Cotati, CA, USA) and peak height was measured on a Philips computing integrator (Model PU 4811, Pye Unicam, Cambridge, UK). Separations were achieved on a reversed-phase C_{18} column (Spheri-5, 5 μm, 220 × 4.6 mm I.D.) preceded by a guard column packed with the same material (30 × 4.6 mm I.D.) (Pierce Chemical Co., Rockford, IL, USA). The mobile phase consisted of phosphate buffer (50 mM, pH 6)–acetonitrile (70:30, v/v) for method 1 and 65:35 (v/v) for method 2. Mobile phase, filtered and degassed before use, was pumped at 1 ml/min flow-rate at ambient temperature for both methods. Retention times of I were 8.0 ± 0.2 min and 5.0 ± 0.2 min in method 1 and method 2, respectively.

2.3. Stock and standard solutions

A stock solution of I (100 μg/ml) was prepared by dissolving 5 mg of I in 50 ml methanol. Working standards were prepared in mobile phase from stock solution in the range of 2.5–1000 ng/ml by sequential dilution method. Calibration samples of I were prepared by adding varying volumes of stock solution of I into dry tubes and evaporating the solvent under a stream of nitrogen gas before adding required volumes of normal rat blood. All the solutions were stored at 4°C.

2.4. Assay procedures

Method 1

To 0.2 ml of drug free or spiked whole blood, 0.6 ml of acetonitrile was added; the tubes were vortex-mixed and kept with occasional vortex-mixing. After 30 min, the tubes were centrifuged at 3000 *g* for 20 min at 0°C and 0.4 ml of the supernatent was evaporated to dryness in a SVC-200H Speed Vac concentrator (Savant Instruments, New York, NY, USA). The residue was reconstituted in 0.1 ml of mobile phase and the entire volume was injected onto the HPLC

through the fixed 50-μl loop. Quantitation was done by the external standard method.

Method 2

To drug free or spiked blood (0.2 ml) was added 2 ml of diethyl ether and the solution was vortex-mixed for 1 min. The tubes were centrifuged at 2500 *g* for 10 min at 0°C. The organic phase was separated by snap freezing the aqueous layer in liquid nitrogen. Snap freezing the aqueous layer was necessary to decant the total volume of ether in order to maximize the recovery of I. Extraction was repeated with another 2 ml of ether to enhance the extraction efficiency. The ether layer was evaporated under reduced pressure in a Savant Vac concentrator and the residue was reconstituted in 0.1 ml of mobile phase and the entire volume was injected onto the HPLC system with the fixed 50-μl loop. The external standard method was used for quantitation.

3. Results and discussion

The HPLC method described here overcomes the problems associated with the previous method, such as lack of sensitivity, introduction of a large amount of endogenous impurities onto the column, possible inter-subject variations and high cost involved in pharmacokinetic studies of I.

The effects of pH and molarity of the buffer salt in the mobile phase on the sensitivity and selectivity of the assay were studied. A decrease in the pH of the mobile phase from 6 to 5, 4, and 3 resulted in decreased sensitivity, while no change in the retention time of I was observed. The reduction in fluorescence at lower pH might be due to the basic nature of I (pK_a 2.08 ± 0.07 [7]). At a lower pH (near pK_a), a higher number of molecules of such a compound exist in the ionised state which has a lower molecular rigidity than the non-ionised form and hence gives a lower fluorescence [8]. A change in the molarity of the buffer salt in the mobile phase did not have significant effect on the peak sharpness. A mobile-phase system containing acetonitrile and dipotassium hydrogen phosphate (pH 6, 50 m*M*) was found to be optimum for proper resolution of I from the endogenous impurities of the blood.

In method 1, the sensitivity of the assay was increased 4-fold by increasing the loop size from 20 μl [6] to 50 μl, minimizing the amount of endogenous impurities injected by increasing the volume of protein precipitant and evaporating the supernatent to dryness, reconstituting the residue in a smaller volume of mobile phase to overcome the dilution effect of acetonitrile, and setting the excitation and emission maxima of the compound at optimum values. The limit of quantitation by this method is 10 ng/ml as compared to 40 ng/ml for the previous method [6].

Calibration curves were obtained by plotting peak heights of I against their corresponding concentrations in spiked blood. Linear least-square regression analysis of the calibration graph demonstrated linearity between the peak height response and the corresponding concentration of I over the range 10–1000 ng/ml. A typical standard curve of I in normal rat blood could be represented by the equation $y = 32.39x - 88.04$. Coefficients of determination were always higher than 0.999.

In method 2, sample preparation was performed by extraction with diethyl ether. Double extraction was essential to achieve maximum recovery of I. Because less impurities were introduced onto the column, which were eluted only up to ca. 3.5 min, the retention time could be reduced to 5 min which resulted in a further increase in sensitivity. The residue, after evaporating the ether layer, was reconstituted in 0.1 ml of mobile phase and due to this two-fold concentration of the sample, the LOQ could be lowered to 2.5 ng/ml in blood. A further advantage of this method was the decrease in analysis time. The standard curve was linear over the range 2.5–1000 ng/ml of I (coefficients of determination > 0.999) and could be represented by the equation $y = 64.15x + 43$. There was no significant change in the slopes over a period of 15 days ($p > 0.05$).

Figs. 2 and 3 show representative chromato-

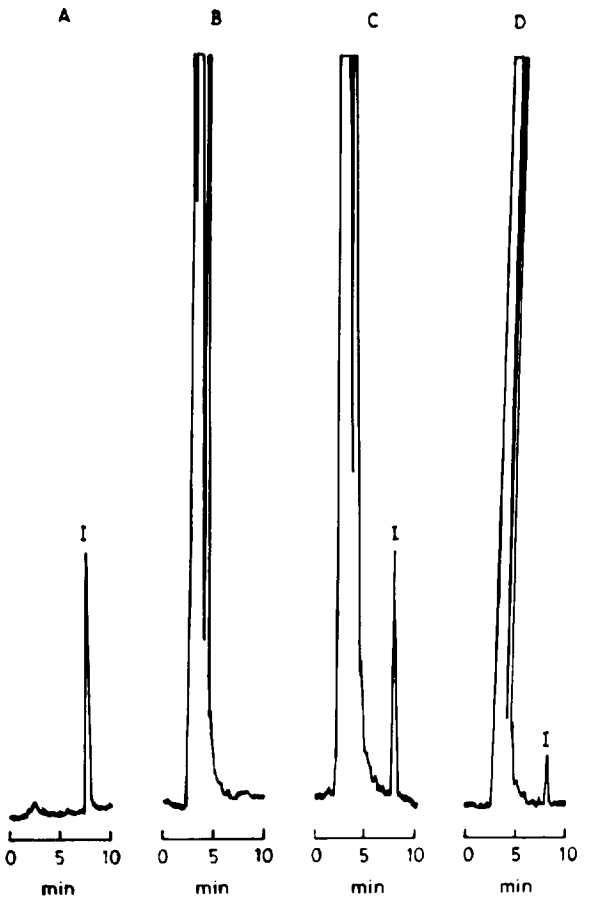

Fig. 2. Chromatograms (method 1) of (A) standard containing 100 ng/ml of I, (B) drug free rat blood, (C) blood containing 100 ng/ml of I, and (D) blood sample of rat taken at 72 h postdose of I.

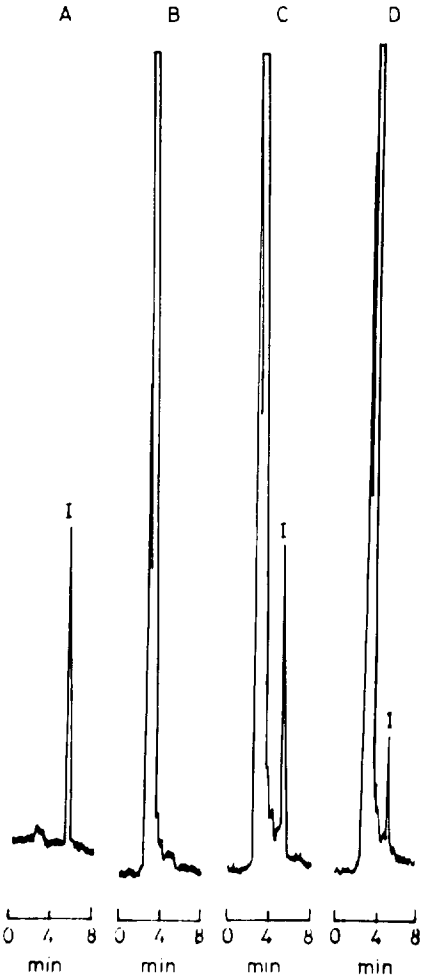

Fig. 3. Chromatograms (method 2) of (A) standard containing 100 ng/ml of I, (B) drug free rat blood, (C) blood containing 50 ng/ml of I after two-fold concentration, and (D) blood sample of rat taken at 72 h postdose of I.

grams of standard containing 100 ng/ml of I (A), drug free rat blood (B), blood standard containing 100 ng/ml of I (method 1) and 50 ng/ml after a two-fold concentration of I (method 2) (C), and blood sample from a rat taken 72 h after administration of a single oral dose of 100 mg/kg of I (D). As evident from the chromatograms, there were no endogenous impurities interfering in the estimation of I in either of the methods.

3.1. Recovery

The recoveries of I from spiked blood samples were calculated by comparing the peak heights at low (LOQ), medium and high concentration levels with those obtained from the analysis of corresponding standard dilutions in mobile phase injected directly. The results are given in Table 1. The recoveries were consistent over the concentration levels considered. The recovery was higher than 100% at the 10 and 2.5 ng/ml levels in methods 1 and 2, respectively. This may be due to magnification of the error in the peak integration and subtle changes in the experimental conditions. It was decided to consider 10 ng/ml and 2.5 ng/ml as the LOQ in methods 1 and 2, respectively, because the coefficients of

Table 1
Absolute recoveries of I from spiked blood samples by methods 1 and 2

Concentration (ng/ml)	Absolute recovery (mean ± S.D.) (%)	
	Method 1 ($n = 5$)	Method 2 ($n = 5$)
2.5	–	100.9 ± 8.4
10	100.9 ± 4.3	–
100	86.0 ± 6.8	86.9 ± 7.9
1000	83.6 ± 2.6	86.2 ± 8.5

variation were within 10% and the signal-to-noise ratio was more than 3.

3.2. Precision of the methods

Validation of the precision of each of the methods was done in terms of the percent relative standard deviations (%R.S.D.) of intra- and inter-assay variations at low (LOQ), medium and high concentrations of I. Percent R.S.D. was calculated according to the equation %R.S.D. = (standard deviation/mean) · 100. An acceptance limit of <15% irrespective of the concentration level was applied [9]. The %R.S.D. values were within acceptable limits for both intra- and inter-day variations (Table 2).

3.3. Accuracy of the methods

Accuracy of each of the methods was assessed by computing the percent deviation of the mean analytical data from the spiked concentration. The acceptance criterion was set at 15% [9]. Percent deviation from actual concentration (%D.F.A.) was calculated by the equation %D.F.A. = [(mean − spiked)/spiked] · 100. The %D.F.A. values in both intra- and inter-day variation studies were within acceptable limits (Table 2).

The HPLC assay method presented here is simple, sensitive and robust. These methods require only 0.2 ml of whole blood, and thus single rat studies could be pursued, minimizing the inter-subject variations and reducing the cost of the experiment during pharmacokinetic studies.

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Table 2
Accuracy and precision of the assay by methods 1 and 2

Method	Concentration (ng/ml)	Inter-assay variation ($n = 5$)		Intra-assay variation ($n = 5$)	
		R.S.D.[a] (%)	D.F.A.[b] (%)	R.S.D. (%)	D.F.A. (%)
1	10	14.5	−1.9	9.5	−13.4
	100	3.5	−0.3	3.8	5.2
	1000	5.1	0.1	0.5	0.5
2	2.5	12.1	−0.8	17.9	7.1
	100	4.2	0.3	3.4	5.4
	1000	0.6	−0.2	1.0	0.4

[a] Percent relative standard deviation.
[b] Percent difference from actual mean.

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