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Simple high-performance liquid chromatography method for the simultaneous determination of ketoconazole and piperine in rat plasma and hepatocyte culture

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Abstract

Piperine, a major alkaloid of black and long peppers has been reported to act as bioavailability enhancer of several drugs by inhibiting drug metabolising enzymes and/or by increasing oral absorption. Ketoconazole is a well established potent inhibitor of CYP 3A4 and P-glycoprotein. A simple and rapid HPLC method has been developed for the simultaneous analysis of ketoconazole and piperine in rat plasma and hepatocyte culture. Analysis was performed using a Symmetry C_{18} column (150×4.6 mm, 5 μm) and isocratic elution with 25 m*M* KH_2PO_4 (pH 4.5)–acetonitrile (50:50) with a flow-rate of 1 ml/min. Photodiode array detection was used to simultaneously monitor piperine at 340 nm and ketoconazole at 231 nm in a single sample. Calibration plots in spiked plasma, hepatocytes and William's medium E were linear over the range studied (10–2000 ng for both drugs). The detection limits for piperine and ketoconazole are 2 and 4 ng, respectively, and the limits of quantitation are 10 and 12 ng, respectively. Intra- and inter-assay variations were less than 8%.

Keywords: Ketoconazole; Piperine; Alkaloids; Enzyme inhibitors

1. Introduction

Black pepper is widely used as spice. It is listed by the US Food and Drug Administration (FDA) as Generally Recognised As Safe (GRAS) and contains 5–9% of the active alkaloid piperine. The latter has been shown to possess several pharmacological actions [1–3]. In addition, it has been reported to enhance the bioavailability of several drugs [4,5]. Recently, it has been patented as bioavailability enhancer [6], as a non-nicotine smoking cessation aid [7], and as an important ingredient of incapacitating composition [8]. It is reported to inhibit drug metabolising enzymes [9]. Considering the fact that it is a constituent of an omnipresent food component, its interactions with other food components and drugs need to be studied. Ketoconazole is a well known potent inhibitor of CYP 3A4 and P-glycoprotein and is hepatotoxic [10]. High-performance liquid chromatography (HPLC) methods for analysis of ketoconazole in human and rat plasma [11,12], human saliva [13] and rat liver, lungs and adrenal glands [14] are reported in the literature. A HPLC method for quantitative analysis of piperine in rat plasma has been described [15]. However, the simultaneous determination of ketoconazole and piperine in rat plasma or hepatocyte culture has not been attempted so far. This communication describes

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a simple, sensitive and rapid HPLC method for simultaneous analysis of piperine and ketoconazole in rat plasma and hepatocyte culture.

2. Experimental

2.1. Chemicals

Potassium dihydrogenphosphate (analytical grade) was from Ranbaxy (Mohali, Punjab, India) and orthophosphoric acid (analytical grade) was from Qualigens (Mumbai, Maharashtra, India). HPLC-grade acetonitrile was from Merck (Mumbai, Maharashtra, India). Piperine (purity >99%, HPLC) was isolated from pepper oleoresin as described earlier [1] while ketoconazole was obtained by extraction of a commercial tablet (Fungicide-200, Torrent, Ahmedabad, India) with dichloromethane (HPLC purity >99%). William's medium E and all other biochemicals for hepatocyte culture except collagenase (Boehringer, Germany) were from Sigma (St. Louis, MO, USA).

2.2. Animals

Male Wistar rats (170±10 g body mass) were obtained from the Institute's animal house kept under standard animal husbandry conditions and pelleted diet. Rats were fasted overnight before the experiment with free access to water.

2.3. Standard plots

Standard plots were prepared by spiking 0.1 ml of rat plasma and $3 \cdot 10^6$ hepatocytes with 10–2000 ng of ketoconazole and piperine whereas 0.1 ml of William's medium was spiked with 20–4000 ng concentration. The samples were processed as described below.

2.3.1. Determination of piperine and ketoconazole in rat plasma

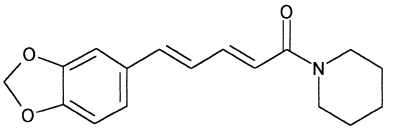
Blood samples (~0.3 ml) were obtained from rats 1 h after the peroral treatment with a combination of 20 mg/kg each of ketoconazole and piperine [as a solution in 5% dimethyl sulfoxide (DMSO) in polyethylene glycol]. Plasma was immediately separated by centrifugation (6000 *g*, 5 min). To 0.1 ml plasma was added 0.3 ml acetonitrile to precipitate the proteins which were separated by centrifugation. The supernatant was then evaporated under a stream of nitrogen at 50°C. The residue was reconstituted in 0.1 ml of solvent system and 50–100 μl of it was injected into the HPLC column. During all the operations samples were protected from light.

2.3.2. Determination of piperine and ketoconazole in rat hepatocyte monolayer culture

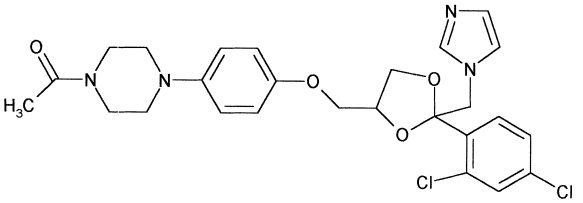
A rat cultured hepatocytes monolayer ($1 \cdot 10^6$ cells/ml) was incubated for 2 h with ketoconazole (10 μ*M*) alone and in the presence of piperine (50 μm). After 2 h, medium was separated and diluted with equal volume of mobile phase, of which 50–100 μl was injected into the system. The cells were extracted with 2 ml methanol followed by centrifugation at 4000 rpm for 5 min. The supernatant was evaporated under a stream of nitrogen at 45°C followed by reconstitution in 100 μl mobile phase of which 50–100 μl was injected.

2.4. Chromatography

A Spectra-Physics (P200) pump was used to deliver isocratically 25 m*M* KH_2PO_4 (pH 4.5, adjusted with orthophosphoric acid)–acetonitrile



Piperine



Ketoconazole

Fig. 1. Chemical structures of piperine and ketoconazole.

Table 1
Equations for standard plots for piperine and ketoconazole in different matrices

Drug	Plasma	William's medium E	Hepatocytes
Piperine	$y=124.9538x+0.0505$ ($r^2=0.9999$)	$y=127.6608x+0.3999$ ($r^2=0.9999$)	$y=124.3843x+0.3323$ ($r^2=0.9999$)
Ketoconazole	$y=25.3350x-0.1375$ ($r^2=0.9995$)	$y=28.5661x-0.2439$ ($r^2=0.9984$)	$y=23.0183x-0.1641$ ($r^2=0.9978$)

$n=5$.

(50:50) at 1 ml/min through a Water's Symmetry C_{18} column (150×4.6 mm, 5 μm) preceded by a guard column of the same material. The samples were injected into the system using a Merck–Hitachi autosampler (Model L7200). An SPD-M6A photodiode array detector set at 340 nm and 231 nm was used to monitor the samples. Data were collected and analysed using computer software ver. 2.24 (Shimadzu, Japan).

3. Results and discussion

Piperine and ketoconazole (Fig. 1) are lipophilic in nature, thus, making it easy to separate them from relatively polar components of plasma, hepatocytes and William's medium E. An equal proportion of acetonitrile and 25 m*M* phosphate buffer (pH 4.5) produced complete separation of the compounds of interest from indigenous components as well as from each other in all the matrices. Using this solvent system piperine appeared at 6.6 min and ketoconazole at 4.6 min. Blank run of all the matrices showed no interference at the retention times of these compounds. Increasing concentration of acetonitrile in the solvent system resulted in the merger of peaks of two drugs together as well as with the endogenous components of the matrices. On the other hand, increase in buffer concentration caused peak broadening. As the majority of compounds are strongly UV absorbing below 300 nm, very less interference from the components of these matrices could be expected at 340 nm. For all the good, no interference were encountered in the analysis of ketoconazole at 231 nm. Standard plots were linear between 10 and 2000 ng. The equations for straight lines for all the matrices are given in Table 1. Detection limits for piperine and ketoconazole were found to be 2 ng and 4 ng, respectively, whereas the limits of quantitation were 10 and 12 ng, respectively. Recovery of both drugs from all the matrices was >98%. Intra- and inter-assay variations were less than 8% and are given in Table 2. The typical chromatograms of blank matrices as well as experimental samples at 231 and 340 nm are given as Figs. 2–4. The plasma sample required for

Table 2
Intra- and inter-assay precisions and accuracies for the determination of piperine and ketoconazole in plasma

	Concentration (ng)	Intra-assay		Inter-assay	
		RSD (%)	Accuracy	RSD (%)	Accuracy
Piperine	10	4.8	104	6.6	101
	400	2.2	101	6.7	105
	2000	2.3	98.3	3.8	100
Ketoconazole	10	7.9	106	7.4	97.1
	400	7.3	97.2	6.9	97.7
	2000	6.2	99.5	6.9	101

$n=5$.

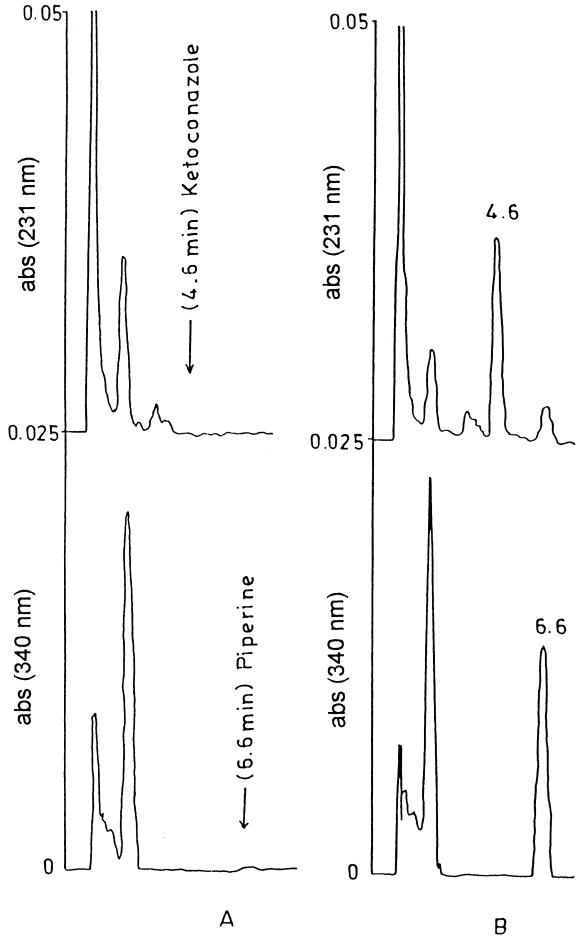


Fig. 2. Chromatogram traces of control plasma (A) and a sample obtained from a rat treated with 20 mg/kg p.o. of piperine+ ketoconazole (B). Piperine was monitored at 340 nm (t_R 6.6 min) and ketoconazole at 231 nm (t_R 4.6 min).

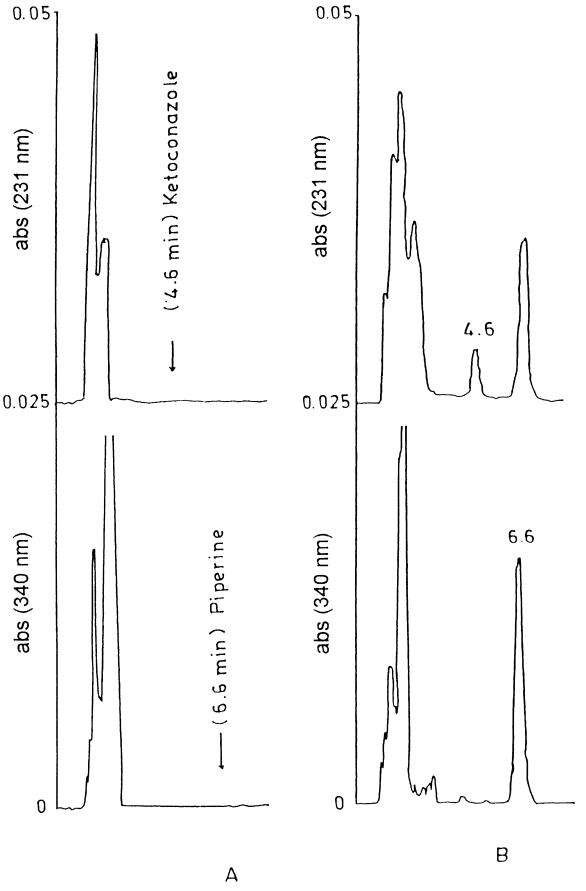


Fig. 3. Chromatogram traces of blank William's medium E (A) and a sample obtained after 2 h incubation of hepatocytes with 25 μM piperine+10 μM ketoconazole (B). Piperine was monitored at 340 nm (t_R 6.6 min) and ketoconazole at 231 nm (t_R 4.6 min).

analysis is small making the method suitable for in-vivo pharmacokinetic studies in small animals where usually there is sample volume limitation. With no interference as well as good separation from endogenous plasma components, this method could be easily modified for analysis of piperine and ketoconazole in the plasma of other animals as well as that of humans. It is obvious that the method is also suitable for analysis of either of the drugs individually using the other drug as an internal standard. Interference from the metabolites of either of the drugs could not be expected due to the fact that relatively polar metabolites would elute with the solvent front.

In conclusion, a simple, sensitive and rapid method has been developed for simultaneous analysis of piperine and ketoconazole in rat plasma and hepatocytes culture.

Acknowledgements

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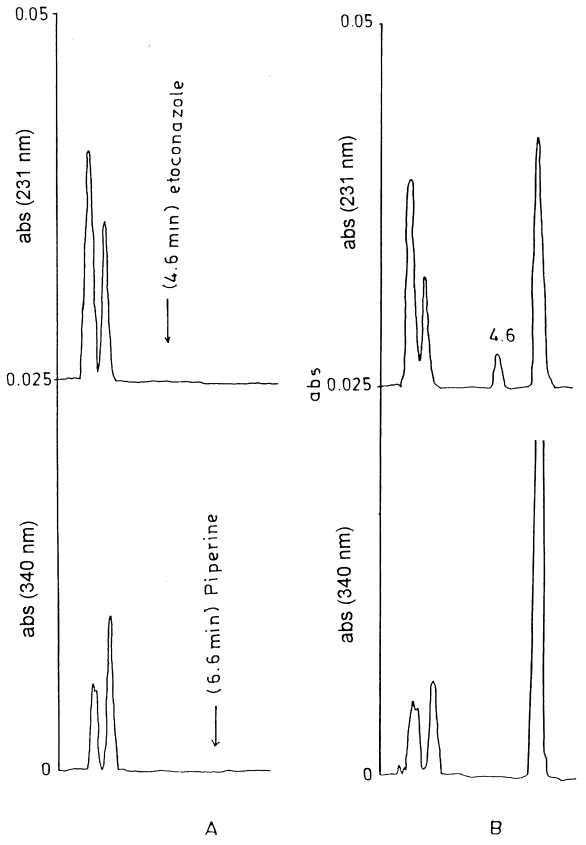

Fig. 4. Chromatogram traces of blank hepatocyte extract (A) and a sample obtained after 2 h incubation of hepatocytes with 25 μ*M* piperine+10 μ*M* ketoconazole (B). Piperine was monitored at 340 nm (t_R 6.6 min) and ketoconazole at 231 nm (t_R 4.6 min).

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