INFLUENCE OF DIOSMIN PRETREATMENT ON THE PHARMACOKINETICS OF CHLORZOXAZONE IN HEALTHY MALE VOLUNTEERS

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SUMMARY

Chlorzoxazone, a centrally acting muscle relaxant, is a probe for cytochrome P450 2E1 (CYP2E1). The first part of the study consisted of oral administration of 250 mg of chlorzoxazone (Paraflex® 250 tablet) alone to 12 healthy male volunteers. Blood samples were collected from the antecubital vein at intervals of 0, 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 hours and urine voided during 0-4 and 4-8 hours was collected after the administration of chlorzoxazone. The second part of the study was conducted after a wash-out period of 7 days; 500 mg of diosmin (Venex®500) was administered daily for 9 days. On day 10, 250 mg of chlorzoxazone was administered. Blood and urine samples were obtained as mentioned above. Serum levels of chlorzoxazone were determined by HPLC. Pharmacokinetic parameters were determined based on non-compartmental model analysis using the computer program RAMKIN. Diosmin pretreatment significantly enhanced AUC, C_{max} and $t_{½}$ with a concomitant reduction in CL/f. The urinary excretion of 6-hydroxychlorzoxazone was decreased and

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unchanged chlorzoxazone was increased over 8 hours. Urinary metabolic ratios of 6-hydroxychlorazoxazone and chlorazoxazone were increased. After pretreatment with diosmin, overall excretion (0-8 h) of 6-hydroxychlorazoxazone and chlorazoxazone were decreased. Diosmin might have inhibited the microsomal CYP2E1-mediated hydroxylation of chlorazoxazone.

KEY WORDS

chlorazoxazone, CYP2E1, diosmin, pharmacokinetics

INTRODUCTION

Chlorzoxazone (Fig. 1), a centrally acting muscle relaxant, is a probe for cytochrome P450 2E1 (CYP2E1) /1/. Chlorzoxazone is almost exclusively metabolized by CYP2E1 to a single major metabolite, 6-hydroxychlorzoxazone, which is rapidly glucuronidated and eliminated by the kidney /2/. Diosmin (3′,5,7-trihydroxy-4′-methoxyflavone 7-rutinoside) is a flavone. Diosmin is used for chronic organic and functional venous insufficiency of the lower limbs, with symptoms such as heavy legs, pain, nocturnal cramps, haemorrhoids and acute haemorrhoidal attacks /3/. Diosmetin, 3′,5,7-trihydroxy-4′-methoxyflavone, is the aglycone of the flavonoid glycoside diosmin. Diosmin and diosmetin are natural dietary agonists of the aryl hydrocarbon receptor (AhR) /4/, reported to potentiate CYP1A1 transcription and its activity. However, diosmetin is only capable of inhibiting CYP1A1 enzyme activity, thus inhibiting carcinogen activation /4/. The pharmacokinetics of diosmin and diosmetin have been extensively studied. Both flavonoids are rapidly metabolized, with diosmetin being partly excreted in bile as glucuronide and sulphate conjugates, while diosmin is partly excreted in the bile unchanged and as the glucuronide conjugate /5/. In earlier studies it was observed that flavonoids inhibit a number of enzymes in the body. We hypothesized that diosmin (a flavone) might interact with chlorzoxazone at the metabolism level. In the present study, we investigated the influence of diosmin pretreatment on the pharmacokinetics of chlorzoxazone in healthy male volunteers.

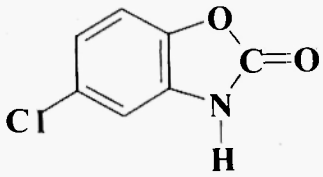


Fig. 1: Structure of chlorazoxazone.

MATERIALS AND METHODS

Materials

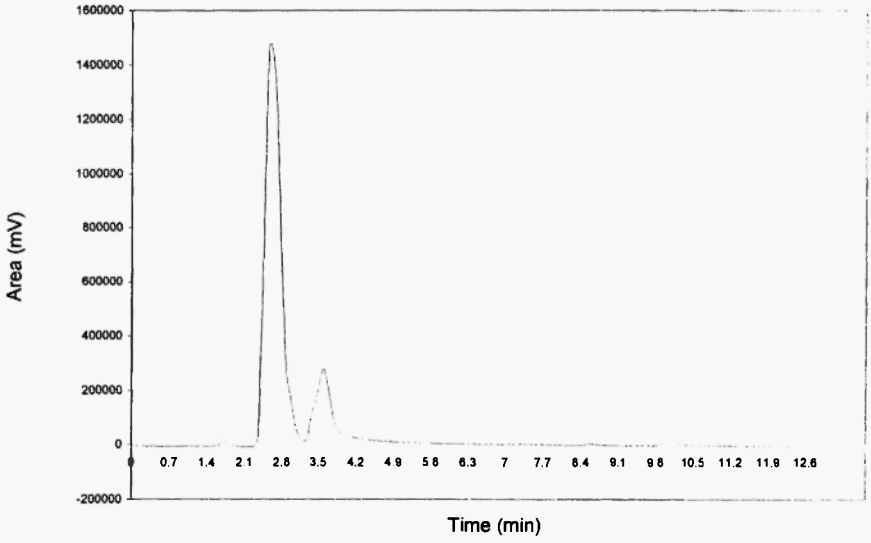
Chlorazoxazone tablets (Paraflex® 250) were purchased from Ethnor Pharmaceuticals Ltd, Mumbai, India. Diosmin tablets (Venex®500) were procured from Elder Pharmaceuticals Ltd, Mumbai, India. Chlorazoxazone pure substance was a kind gift from Biological-E Ltd, Hyderabad, India. Phenacetin pure substance was a kind gift from Dr. Reddy's Research Foundation, Hyderabad, India. Acetonitrile, methanol (HPLC grade) and glacial acetic acid (AR grade) were purchased from E. Merck Ltd, Mumbai, India.

High performance liquid chromatography (HPLC) system

A Shimadzu HPLC unit equipped with LC-8A solvent delivery module, SPD-10AVP UV-visible spectrophotometer detector, Class CR-10 data processor, rheodyne (with 20 µl capacity loop) injection port and Wakosil II C-18 column (stainless steel column of 25 cm length and 4.6 mm internal diameter packed with porous silica spheres of 5 µ diameter, 100 Å pore diameter) was used for analysis of samples. The mobile phase consisted of acetonitrile:0.5% glacial acetic acid (40:60 v/v) with a flow rate of 1 ml/min. The eluent was monitored at 287 nm and sensitivity of 0.001 a.u.f. was used for analysis.

Study design

Healthy volunteers were informed about the study and written consent was obtained. The volunteers had no history of ill health during the preceding six months and none had taken any medication



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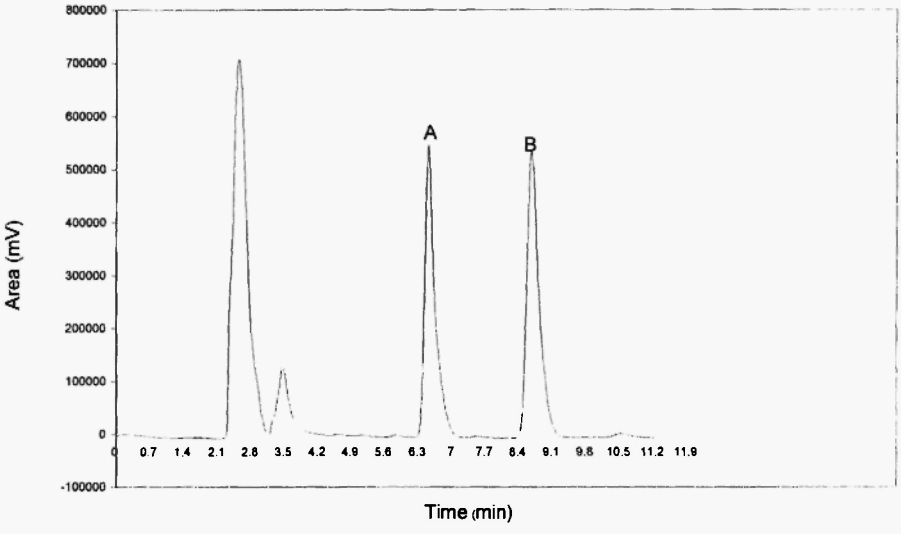

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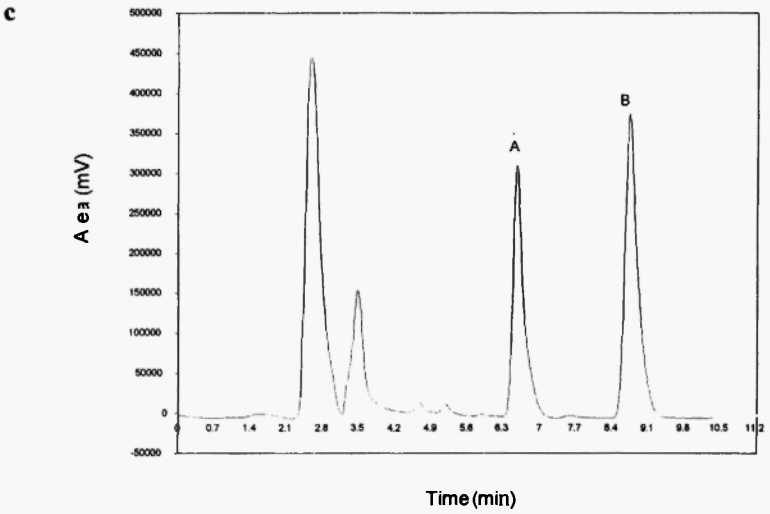

Fig. 2: **a:** Serum blank chromatogram; **b:** serum standard chromatogram (A = phenacetin, B = chlorazoxazone); **c:** serum test chromatogram (A = phenacetin, B = chlorazoxazone).

for at least 15 days prior to the administration of chlorzoxazone in this study. The local ethics committee approved the study protocol. Study drugs were taken in the morning with 100 ml of tap water just after voiding. Twelve healthy male volunteers with a mean age of 25 ± 3 years (22-28 years), a mean height of 172.4 ± 5.0 cm (165-180 cm) and a mean body weight of 61.8 ± 6.6 kg (54-70 kg) participated in the study after undergoing a thorough physical examination. The study was conducted at the University College of Pharmaceutical Sciences, Kakatiya University, Warangal, Andhra Pradesh, India.

The first part of the study consisted of oral administration of 250 mg of chlorazoxazone alone. Blood samples were obtained from the antecubital vein after 0, 0.5, 1, 2, 3, 4, 5, 6, 7 and 8 hours, and urine voided during 0-4 and 4-8 hours was collected after the administration of chlorzoxazone. The second part of the study was conducted after a wash-out period of one week. 500 mg of diosmin was administered daily for 9 days. On day 10, a 250 mg tablet of chlorzoxazone was administered. Blood (2 ml) and urine (5 ml) samples were collected as described above. Blood samples were centrifuged at 3,000 rpm for 15 minutes. Serum and urine samples were stored at -80°C until analysis.

Method of analysis

Chlorzoxazone in serum samples was estimated by reversed-phase HPLC /6/. To 250 µl of serum, 10 µl of internal standard phenacetin (1 mg/ml) was added and vortexed for 2 min. An equal volume of methanol was added to serum samples for protein precipitation, vortexed for one minute and centrifuged at 13,000 rpm for 8 min using a Biofuge Fresco centrifuge (Heraeus, Germany). Urinary metabolite levels were estimated by taking 100 µl of urine and diluting the sample five times with deionized water. 20 µl of the supernatant (both serum and urine) were injected onto the HPLC column. A linearity calibration curve in the range of 2-36 µg/ml was established (r^2 = 0.999) in serum matrix. The retention times of chlorzoxazone and internal standard were 9.2 and 6.8 minutes, respectively (Fig. 2b,c). A blank chromatogram of chlorzoxazone is shown in Figure 2a.

Statistical analysis

Pharmacokinetic parameters - area under the curve (AUC), elimination half-life ($t_{½}$), apparent volume of distribution (Vd/F) and apparent systemic clearance (CL/f) - were calculated for each subject using a non-compartmental pharmacokinetic program RAMKIN /7/. This program is based on statistical moments theory. It has been validated by comparing the outputs with those from WinNonlin.

RESULTS

The means ± standard deviation (SD) serum concentrations of chlorazoxazone at different time points before and after diosmin pretreatment are shown in Figure 3. The pharmacokinetic parameters of chlorazoxazone before and after diosmin pretreatment are presented in Table 1. Intra- and inter-day precision of determination of chlorzoxazone in human serum are given in Table 2. In the present study, it was observed that pretreatment with diosmin for 8 days resulted in an increase in serum chlorzoxazone levels. There was no significant change in t_{max} of chlorzoxazone in the volunteers following diosmin pretreatment. Diosmin pretreatment significantly increased the $AUC_{(0-8)}$ and C_{max} of chlorzoxazone by 39% and 34%, respectively, with a concomitant decrease in clearance by 40% and increase in half-life by 20%. The mean ± SD urinary recovery of the metabolite (6-

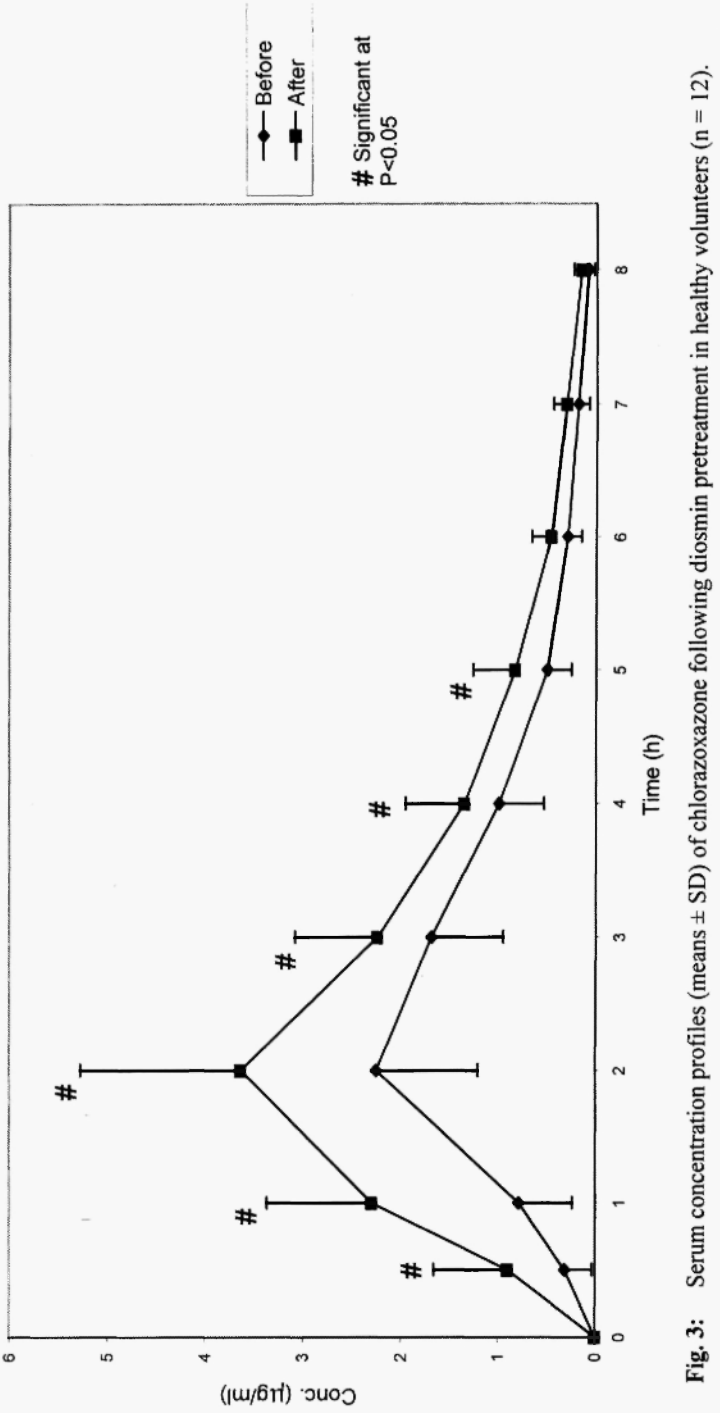

Fig. 3: Serum concentration profiles (means ± SD) of chlorazoxazone following diosmin pretreatment in healthy volunteers (n = 12).

TABLE 1

Pharmacokinetic parameters of chlorzoxazone in human serum before and after pretreatment with diosmin

	Before	After	p value
C_{max} (μg/ml)	2.408 ± 0.99	3.675 ± 1.62	<0.002
t_{max} (h)	2.166 ± 0.38	2.0 ± 0.42	NS
$AUC_{(0-8)}$ (μg/ml/h)	6.71 ± 2.18	11.08 ± 3.79	<0.00004
$AUC_{(0-\infty)}$ (μg/ml/h)	6.88 ± 2.18	11.408 ± 3.73	<0.00002
$t_{½}$ (h)	1.15 ± 0.42	1.43 ±0.57	<0.042
CL/f (ml/h/kg)	658.85 ± 251.82	393.45 ± 132.35	<0.00002
Vd/f (ml//kg)	847.61 ± 851.75	610.10 ± 610.97	<0.03

Values are means ± SD (n = 12).
$AUC_{(0-\infty)}$ = area under the time-concentration curve from 0 to infinity; $AUC_{(0-8)}$ = area under the time-concentration curve from 0-8 h; CL/f = apparent systemic clearance; C_{max} = peak serum concentration; t_{max} = time to reach C_{max}; $t_{½}$ = elimination half-life; Vd/f = apparent volume of distribution; SD = standard deviation; NS = not significant.

hydroxychlorzoxazone) and unchanged chlorzoxazone during 0-8 hours is given in Table 3. The urinary excretion of 6-hydroxychlorzoxazone was decreased by 32% whereas the excretion of unchanged chlorzoxazone was increased by 50% over 8 hours following diosmin pretreatment.

DISCUSSION

The apparently decreased metabolism of chlorzoxazone and decreased excretion of 6-hydroxychlorzoxazone and increased excretion of unchanged chlorzoxazone after pretreatment with diosmin may be due to decreased expression of CYP2E1 in the liver /8/. The 6-hydroxylation of chlorzoxazone by human liver microsomes has been shown to be primarily mediated by CYP2E1 /9/. Induction of CYP2E1 explains the increased vulnerability of the heavy alcohol consumer to the toxicity of substances such as isoniazid, pyridine, phenyl butazone

TABLE 2

Intra- and inter-day precision of determination of chlorzoxazone in human serum

Spiked concentration (μg/ml)	Day	Mean concentration (μg/ml)		
		Mean	**SD**	**RSD**
Inter-day variation (n = 6)				
1	**0**	1.09	0.03	2.752
	1	0.99	0.02	2.02
	2	0.97	0.05	5.154
	3	0.99	0.04	4.04
	4	0.98	0.03	3.061
10	**0**	10.91	0.10	0.916
	1	10.12	0.23	2.272
	2	10.85	0.30	2.765
	3	10.63	0.13	1.223
	4	9.98	0.27	2.705
50	**0**	49.78	1.62	3.25
	1	51.09	2.01	3.934
	2	50.18	1.98	3.945
	3	50.98	1.72	3.373
	4	50.72	2.08	4.101
100	**0**	102.38	1.98	1.934
	1	100.97	2.30	2.278
	2	103.09	2.69	2.609
	3	99.03	1.69	1.706
	4	100.67	2.98	2.960
Intra-day variation (n = 16)				
1		0.99	0.03	3.03
10		10.63	0.19	1.787
50		51.01	0.32	0.627
100		101.92	0.78	0.765

RSD = relative standard deviation.

TABLE 3

Urinary excretion of chlorzoxazone and 6-hydroxychlorzoxazone collected over 8 hours following diosmin pretreatment

	Before	After	p value
6-Hydroxychlorzoxazone (mg/ml)	2.58 ± 1.17	1.746 ± 02.30	<0.005
Chlorzoxazone (mg/ml)	1.792 ± 0.88	3.59 ± 2.57	<0.0006

Values are means ± SD (n = 12).

and acetaminophen (paracetemol) which are specific inducers of CYP2E1 /10/.

Diosmin exhibits potent inhibition of ethoxyresorufin-*O*-deethylase (EROD) activity by 11% at 0.25 mM concentration and by 61% at 0.5 mM. It inhibits methoxyresorufin-*O*-demethylase (MROD) by 47% and 54% at the two concentrations tested but did not significantly alter benzyloxyresorufin-*O*-dealkylase (BROD) activity. The alkoxyresorufin-*O*-dealkylase reactions are selective for various isoforms of cytochrome P450 and therefore diosmin might have varied effects on the metabolism of substrates for these isoforms /11/. Quantification of CYP2E1-dependent hydroxylation of chlorzoxazone has been used to characterize specific enzyme activities. Induction of CYP2E1 occurs in the first month after surgery in liver transplant patients, and drugs that are substrates for CYP2E1 may require dosage alteration during that period /12/. The results of the present investigation suggest an increase in AUC and a decrease in CL/f of chlorzoxazone after diosmin pretreatment due to CYP2E1 inhibition.

CONCLUSION

From the results of the present study, we suggest that the decreased metabolism of chlorzoxazone by diosmin pretreatment is due to CYP2E1 inhibition. It has been suggested that CYP2E1 inhibitors may eventually provide useful tools for the prevention and treatment of the hepatotoxicity associated with heavy alcohol drinking and protection against cancer.

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