

**DEVELOPMENT AND VALIDATION
OF A STABILITY-INDICATING RP-HPLC METHOD
FOR ANALYSIS OF DOXOFYLLINE
IN HUMAN SERUM. APPLICATION OF THE METHOD
TO A PHARMACOKINETIC STUDY**

R. Gannu¹, S. Bandari¹, S. G. Sudke¹, Y. M. Rao^{1,}, and B. P. Shankar²*

¹Centre for Biopharmaceutics and Pharmacokinetics, University College
of Pharmaceutical Sciences, Kakatiya University, Warangal, Andhra Pradesh, India

²Euro Drugs India, Hyderabad, India

SUMMARY

A simple, isocratic, stability-indicating reversed-phase high-performance liquid chromatographic (RP-HPLC) method has been developed for determination of doxofylline in human serum. The method employs precipitation for isolation and sample concentration, followed by reversed-phase liquid chromatographic analysis with ultraviolet (UV) detection at 275 nm. Analytes were extracted from serum with 400 μL methanol. Samples containing the internal standard (metronidazole) and doxofylline were chromatographed on a C_{18} column with 18:82 acetonitrile–phosphate buffer (12.5 mM potassium dihydrogen orthophosphate, pH 3.0) as mobile phase at a flow rate of 1 mL min^{-1} . The retention times of doxofylline and metronidazole were 7.54 min and 4.36 min, respectively. The intra-day and inter-day coefficients of variation and the error of the assay were $<15\%$. The calibration plot was linear for concentrations in the range of 0.025 to $25 \mu\text{g mL}^{-1}$. The extraction recovery of doxofylline was $>95\%$. The validated method was applied to a pharmacokinetic study of doxofylline in human serum after administration of a single doxofylline tablet (400 mg). The method is ideally suited to estimation of the drug for pharmacokinetic studies on human volunteers after oral administration of doxofylline.

INTRODUCTION

Doxofylline (7-(1,3-dioxalan-2-ylmethyl)theophylline; Fig. 1) is a bronchodilator xanthine drug which has the therapeutic properties of theophylline with lower incidence of side-effects [1,2]. It has been reported to

have a better safety profile, because of reduced affinity for adenosine A1 and A2 receptors [3–6]. Moreover, unlike theophylline, doxofylline does not antagonize calcium-channel-blocker receptors nor does it interfere with the influx of calcium into cells [7]. Doxofylline (DOX) is used in the treatment of bronchial asthma, chronic obstructive pulmonary disease (COPD), and chronic bronchitis.

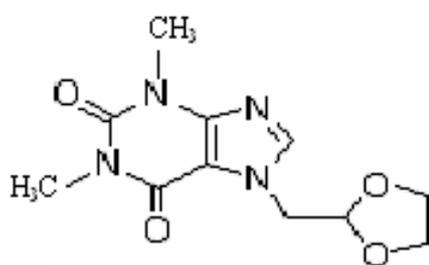


Fig. 1

The structure of doxofylline

Assays have been reported for quantification of doxofylline in biological samples, plasma [8], or serum [9,10]. These techniques use solid-phase extraction [8,10] for isolation of the drug, or a non-extraction direct-injection HPLC method [10]; such methods are not economically feasible for routine use in pharmacokinetic studies in which numerous samples must be analysed. In the non-extraction direct-injection HPLC method, strong protein binding could theoretically hamper or disturb the absorption equilibria of the analytes [11]. The linear ranges of reported methods vary from 0.03–10 mg L⁻¹ [8] to 6–100 mg L⁻¹ [10].

A simple, sensitive, reliable, and cost-effective, RP-HPLC method, validated in accordance with International Conference on Harmonization (ICH) guidelines [12,13], for determination of doxofylline in human serum is described in this paper. The advantages of the method include small sample volume, single-step liquid–liquid extraction with methanol, and short run time. Protein precipitation was selected because it has obvious advantages, for example shorter processing time, consumption of less organic solvent, fewer steps, and good clean up of plasma samples. We also show the suitability of the method for a pharmacokinetic study with human volunteers.

EXPERIMENTAL

Materials

Pure samples of doxofylline and metronidazole were gifts from Euro Drugs, Hyderabad, India and Torrent Pharmaceuticals, India, respectively. Acetonitrile and methanol (HPLC grade) were obtained from Rankem, India. Potassium dihydrogen orthophosphate (AR grade) was obtained from Hi Media, India. Double-distilled water was used throughout the HPLC procedure.

Preparation of Calibration Standards and Quality-Control (QC) Samples

Stock solutions (1.0 mg mL^{-1}) of doxofylline and metronidazole were prepared in methanol. Working solutions (100.0 and $1.5 \text{ } \mu\text{g mL}^{-1}$) were prepared by appropriate dilution of the stock solutions of doxofylline and metronidazole, respectively. Doxofylline working solution was used to prepare spiking stock solutions for construction of eight-point calibration plots (0.025 – $25 \text{ } \mu\text{g mL}^{-1}$) and QC samples at four different levels (0.025 , 5.0 , 10.0 , and $25.0 \text{ } \mu\text{g mL}^{-1}$). All stock solutions were kept refrigerated (4°C) when not in use. Calibration standards and QC samples were prepared in bulk by spiking $500 \text{ } \mu\text{L}$ control serum with $100 \text{ } \mu\text{L}$ respective spiking stock solution; they were then divided into smaller volumes and stored at -20°C until analysis.

Sample Preparation for Analysis

Serum containing doxofylline ($500 \text{ } \mu\text{L}$) was pipetted into microtubes, $100 \text{ } \mu\text{L}$ internal standard solution ($1.5 \text{ } \mu\text{g mL}^{-1}$ metronidazole) was added, and the sample was vortex mixed for 2 min. Methanol ($400 \text{ } \mu\text{L}$) was added to precipitate serum proteins, and the sample was vortex mixed for 8 min and centrifuged at 3500 rpm for 10 min. Supernatant ($400 \text{ } \mu\text{L}$) was transferred to a glass tube and evaporated in a vacuum oven (Sheldon Manufacturing, USA) at 40°C . The residue was reconstituted in $200 \text{ } \mu\text{L}$ mobile phase and $20 \text{ } \mu\text{L}$ of the reconstituted sample was injected for HPLC analysis.

Chromatographic Conditions

HPLC was performed with an LC-10AT solvent module and SPD-10A variable-wavelength UV–visible spectrophotometric detector with LC 10 software, all from Shimadzu (Japan). Compounds were separated on a $150 \text{ mm} \times 4.6 \text{ mm i.d.}$, $5\text{-}\mu\text{m}$ particle, Inertsil C_{18} analytical column. The

mobile phase was 18:82 acetonitrile–12.5 mM potassium dihydrogen orthophosphate buffer (pH adjusted to 3.0 with orthophosphoric acid) at a flow rate of 1 mL min⁻¹. The injection volume was 20 µL, the elute was monitored at 275 nm, and the sensitivity was 0.0007 AUFS.

Assay Validation

Intra-run and inter-run precision and accuracy of the assay ($n = 5$) were determined as *CV* (%) and error (%), respectively, on the basis of published guidelines [14]. Samples spiked with 0.025, 5.0, 10.0, and 25.0 µg mL⁻¹ were analysed. Five replicates at each concentration were processed as described above, on days 1, 3, 5, and 10, to determine intra day and inter day precision and accuracy. Percentage error was calculated by use of the equation:

$$\text{Error (\%)} = \frac{\text{Calculated concentration} - \text{Added concentration}}{\text{Added concentration}} \times 100$$

Limits of Detection and Quantification

The limit of detection (*LOD*) is the smallest amount of a sample that can be differentiated from background noise but not quantified. *LOD* was determined as the amount for which the signal-to-noise ratio (*S/N*) was 3:1 by comparing results from samples of known concentration with results from blank samples. The limit of quantification (*LOQ*) was defined as the lowest concentration of analyte that could be determined with acceptable precision and accuracy and was established by determining the concentrations of four spiked calibration standards.

Extraction Efficiency

Recovery of doxofylline was determined for QC samples at concentrations of 0.025, 5.0, 10.0, and 25.0 µg mL⁻¹. Five replicates of each QC sample were extracted as described above and analysed by HPLC. The extraction recovery at each concentration was calculated by use of the equation:

$$\text{Recovery (\%)} = \frac{\text{Peak area after extraction}}{\text{Peak area after direct injection}} \times 100$$

Stability Studies

To ensure the reliability of the results in relation to handling and

storage of serum samples and stock standard solutions, stability studies were performed at four different concentrations (0.025, 5.0, 10.0, and 25.0 $\mu\text{g mL}^{-1}$). The long-term stability of doxofylline in human serum was assessed by analysis after storage for 30 days at -20°C . Freeze–thaw stability was determined at 0.025, 5.0, and 10.0 $\mu\text{g mL}^{-1}$ through three cycles of thawing at room temperature for 2–8 h and then refreezing at -20°C for 12–24 h. Stability in spiked human serum (0.025, 5.0, and 10.0 $\mu\text{g mL}^{-1}$) at room temperature (bench-top stability) was evaluated for 8 h by comparison with freshly prepared extracted samples. The stability of stock solution of doxofylline and the IS was determined at room temperature for 8 h and under refrigeration ($2\text{--}8^{\circ}\text{C}$) for 15 days. The concentration of doxofylline after each storage period was related to the initial concentration as determined for freshly prepared samples.

Application to Pharmacokinetic Study

Six healthy human volunteers (body weight 66.2 ± 6.7 kg) fasted for 12 h before administration of the drug. Blood samples (5 mL) were withdrawn from the ante cubital vein 0, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 12.0, 16.0, and 24.0 h after single oral administration of a doxofylline tablet (400 mg). All blood samples were allowed to clot and then centrifuged for 10 min at 3000 rpm. The serum was transferred to clean tubes and stored at -20°C until HPLC analysis.

Pharmacokinetic data peak serum concentration (C_{Max}), time to reach peak concentration (t_{Max}), area under the serum-concentration–time curve (AUC), and elimination half-life ($t_{1/2}$) for doxofylline were obtained for each subject using the computer software Kinetica (Innaphase, 1999) intended for calculation of model-independent data.

RESULTS AND DISCUSSION

Chromatography

Representative chromatograms obtained from blank serum, a spiked serum sample, and a serum sample from the pharmacokinetic study are shown in Figs 2–4. The doxofylline and internal standard (IS) peaks were resolved with good symmetry; the retention times were 7.54 and 4.36 min, respectively. No endogenous interfering peaks were observed in blank serum at the retention times of doxofylline and metronidazole, confirming the specificity of the method. System suitability data for the method were: num-

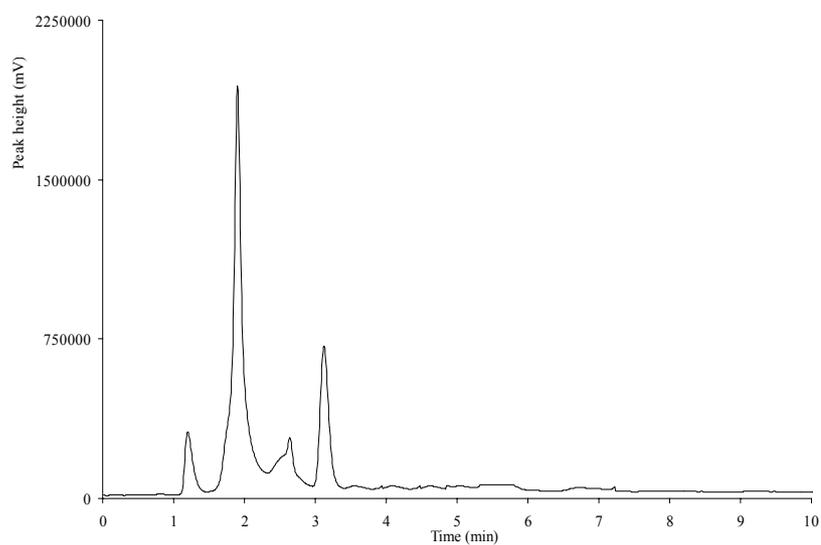


Fig. 2
Typical chromatogram obtained from blank serum

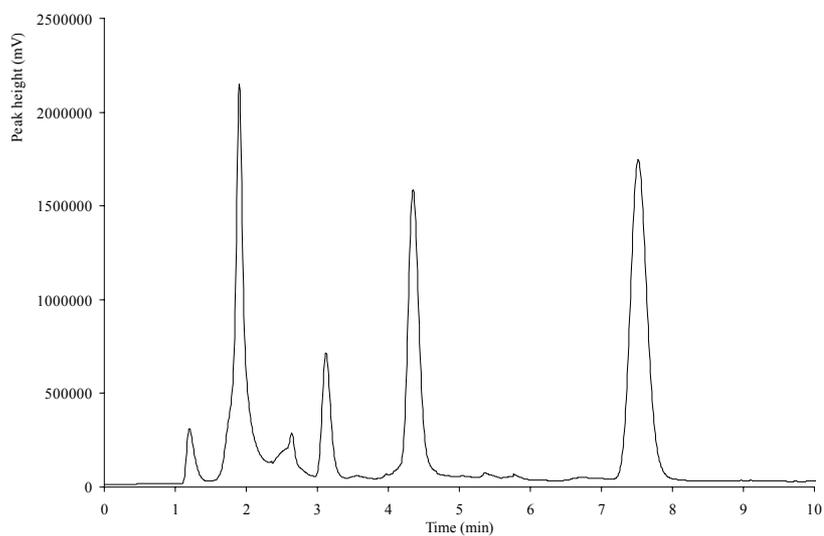


Fig. 3
Typical chromatogram obtained from serum sample spiked with doxofylline. The retention times of doxofylline and metronidazole (IS) are 7.54 and 4.36 min

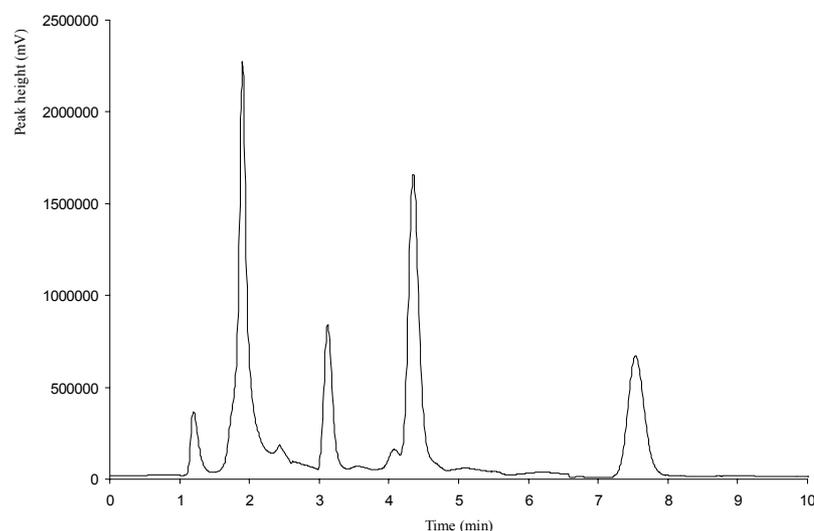


Fig. 4

Typical chromatogram obtained from a serum sample from a subject 16 h after dosing. The retention times of doxofylline and metronidazole (IS) are 7.56 and 4.36 min. The concentration of doxofylline was $0.27 \mu\text{g mL}^{-1}$

number of theoretical plates 1262 and 1172 m^{-1} for doxofylline and the IS, respectively; tailing factors <1.1 for both doxofylline and IS, and resolution between doxofylline and the IS 0.32.

Quantification

The doxofylline-to-IS peak-area ratio was used for quantification of doxofylline in serum. The calibration plot was linear in the concentration range 0.025 to $25 \mu\text{g mL}^{-1}$. If the calibration/regression equation is given by $y = mx + c$, where y represents the doxofylline-to-IS peak-area ratio, x represents the concentration of doxofylline, m is the slope of the plot, and c is the intercept, the equation obtained from the eight-point calibration plot was $y = 0.1404x - 0.0023$ ($r^2 = 0.9978$). The limit of quantification (LOQ) was $0.025 \mu\text{g mL}^{-1}$ and reproducible, with a relative standard deviation (RSD) less than 20% and accuracy of 80 to 120%. The limit of detection (LOD) was $0.010 \mu\text{g mL}^{-1}$, on the basis of a signal-to-noise (S/N) ratio of 3:1. The intra-day precision of the assay was determined by analysis of five spiked serum samples at each concentration (0.025 , 5 , 10 , and $25 \mu\text{g mL}^{-1}$) on the same day. For determination of inter-day precision, the samples we-

re analysed on four different days. The intra-day and inter-day coefficients of variation (*CV*, %) and error (%) values are shown in Table I. These values were within limits (<15%) specified for inter-day and intra-day precision [15,16]. Recovery of doxofylline using the procedure was consistent and efficient. The mean absolute recovery (Table II) of doxofylline at 0.025, 5, 10, and 25 $\mu\text{g mL}^{-1}$ was 95.86, 98.99, 99.35, and 100.30%, respectively. Recovery of metronidazole was also determined and found to be $93.58 \pm 1.28\%$.

Table I

Intra-day and inter-day accuracy and precision of the assay ($n = 5$)

Conc. added ($\mu\text{g mL}^{-1}$)	Calculated concentration ($\mu\text{g mL}^{-1}$)		% <i>CV</i>		% Error	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
0.025	0.025	0.026	8.00	13.46	0.00	4.00
5	5.05	4.98	4.22	7.59	0.90	-0.40
10	10.04	10.09	4.94	5.06	0.40	0.90
25	25.08	24.95	4.90	4.49	0.34	-0.20

Table II

Recovery and accuracy of the method

Conc. ($\mu\text{g mL}^{-1}$)	Absolute recovery			Accuracy (%)		
	Mean \pm <i>SD</i> ($n = 5$)	Range (min-max)	<i>CV</i> (%)	Mean \pm <i>SD</i> ($n = 5$)	Range (min-max)	<i>CV</i> (%)
0.025	95.86 ± 8.27	89.26–102.09	8.62	96.43 ± 11.52	83.65–106.02	11.95
5	98.99 ± 6.33	91.13–105.21	6.39	98.65 ± 4.62	96.25–100.23	4.68
10	99.35 ± 7.39	92.18–104.20	7.46	100.00 ± 8.22	92.15–108.56	9.13
25	100.30 ± 4.64	95.34–104.71	4.62	99.99 ± 6.39	92.07–106.29	6.39

Stability

A stock solution containing $5 \mu\text{g mL}^{-1}$ was analysed. After storage for 15 days at $2-8^{\circ}\text{C}$ and at room temperature for 8 h, more than 98% of the doxofylline remained unchanged, on the basis of comparison of peak areas with those obtained from a freshly prepared solution of doxofylline ($5 \mu\text{g mL}^{-1}$). This suggests doxofylline standard solution was stable for at least 15 days when stored at $2-8^{\circ}\text{C}$ and for 8 h at room temperature. Bench-

top stability of doxofylline in serum was investigated at concentrations of 0.025, 5, and 10 $\mu\text{g mL}^{-1}$; the results revealed that doxofylline in serum was stable for at least 8 h at room temperature with average percentage recovery of 97.12, 98.81, and 98.41% respectively. During repeated freezing and thawing (three cycles) of serum samples spiked with doxofylline at three levels (0.025, 5, and 10 $\mu\text{g mL}^{-1}$), mean recovery was 100.00, 98.82, and 99.60%, respectively. Long-term stability of doxofylline in serum at -20°C was also determined by analysis after 30 days of storage at four concentrations (0.025, 5, 10, and 25 $\mu\text{g mL}^{-1}$); mean recovery was 100.00, 99.60, 98.61, and 98.41%, respectively. The results from the stability tests (Table III) indicated doxofylline was stable under the conditions studied.

Table III

Results from study of the stability of doxofylline

Stability	Concn added	Ave calculated concentration of comparison sample ($\mu\text{g mL}^{-1}$) ^d	Ave calculated concentration of stability sample ($\mu\text{g mL}^{-1}$) ^d	Ave recovery (%)
Bench-top ^a	0.025	0.026 \pm 0.002	0.025 \pm 0.003	97.12
	5	5.05 \pm 0.07	4.99 \pm 0.208	98.81
	10	10.01 \pm 0.281	9.89 \pm 0.411	98.41
Freeze and thaw ^b	0.025	0.025 \pm 0.002	0.025 \pm 0.002	100.00
	5	5.07 \pm 0.079	5.01 \pm 0.357	98.82
	10	10.04 \pm 0.172	10.00 \pm 0.283	99.60
Long term ^c	0.025	0.025 \pm 0.002	0.025 \pm 0.004	100.00
	5	5.02 \pm 0.070	5.00 \pm 0.158	99.60
	10	10.06 \pm 0.146	9.92 \pm 0.463	98.61
	25	25.09 \pm 0.725	24.69 \pm 0.822	98.41

^aAfter 8 h at room temperature

^bAfter three freeze–thaw cycles

^cAfter 30 days at -20°C

^dValues are mean \pm SD ($n = 3$)

Application to Pharmacokinetic Study

The method was used for analysis of serum samples obtained after oral administration of a single doxofylline tablet (dose 400 mg) to six healthy human volunteers. Figure 5 shows the mean serum concentration–time curve for doxofylline. Pharmacokinetic data, estimated by use of Kinetica software, are shown in Table IV. A peak concentration (C_{Max} , mean \pm SD) of $3.48 \pm 0.477 \mu\text{g mL}^{-1}$ doxofylline was reached after $1.50 \pm 0.50 \text{ h}$ (t_{Max} ,

mean \pm *SD*). The half-life was 5.29 ± 1.112 h. The area under the serum concentration–time curve (AUC_{0-24}) was $19.29 \pm 2.364 \mu\text{g h mL}^{-1}$. These results are comparable with those reported elsewhere [9,17].

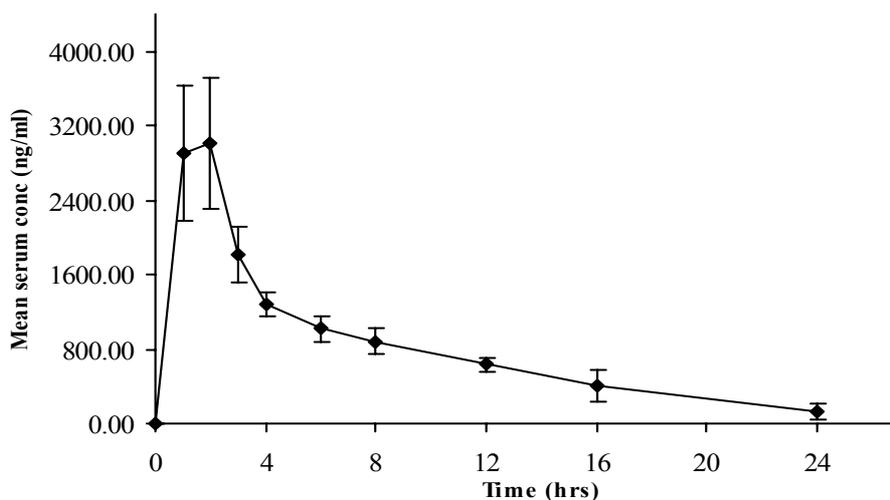


Figure 5

Mean (\pm *SD*) concentration–time profile for doxofylline in the serum of healthy volunteers ($n = 6$) after administration of a single 400-mg doxofylline tablet

Table IV

Pharmacokinetic data for doxofylline

Data	Value
C_{Max} ($\mu\text{g mL}^{-1}$)	3.48 ± 0.477
t_{Max} (h)	1.50 ± 0.5
$AUC_{(0-24)}$ ($\mu\text{g h mL}^{-1}$)	19.29 ± 2.364
$AUC_{(\text{Total})}$ ($\mu\text{g h mL}^{-1}$)	20.54 ± 2.873
$t_{1/2}$ (h)	5.29 ± 1.112

CONCLUSIONS

A simple, sensitive, economic, and reliable RP-HPLC method has been developed and validated for determination of doxofylline in human serum over the concentration range $0.025\text{--}25 \mu\text{g mL}^{-1}$. The method entails sample preparation by protein precipitation, extraction with methanol,

then chromatographic separation with UV detection. No interfering peaks were observed at the elution times of doxofylline and the IS. The method is accurate, reproducible, specific, rapid (run time 10 min), requires relatively small volumes of serum (0.5 mL), and is applicable to evaluation of pharmacokinetic profiles of doxofylline in human volunteers. The method is suitable for routine analysis of doxofylline in human serum in bioavailability and bioequivalence studies.

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