Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
33 (2003) 755–764
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

HPLC-UV method development and validation for 16-dehydropregnenolone, a novel oral hypolipidaemic agent, in rat biological matrices for application to pharmacokinetic studies☆

S.K. Singh, Nitin Mehrotra, S. Sabarinath, R.C. Gupta *

Pharmacokinetics and Metabolism Division, Central Drug Research Institute, Chattar Manzil Palace, P.O. Box No. 173, Lucknow 226001, India

Received 5 March 2003; received in revised form 27 May 2003; accepted 28 May 2003

Abstract

An accurate and precise HPLC assay has been developed and validated for determination of dehydropregnenolone (DHP) in rat plasma, bile, urine and feces. Separation was achieved using a C-18 reversed phase column with a mobile phase comprising of acetonitrile and deionized water (55:45% v/v) using a UV detector, set at a wavelength of 248 nm. The method, applicable to 200-μl plasma, bile and urine, involved double extraction of the samples with *n*-hexane. The sample clean up for feces involved single extraction of 50 mg of sample with 3 ml of acetonitrile. The method was sensitive with a limit of quantitation of 20 ng/ml in all the matrices and absolute recovery $>92\%$. Precision and accuracy were within the acceptable limits, as indicated by relative standard deviation varying from 4.7 to 11.2% and bias values ranging from 1.8 to 8.8%. Moreover, DHP was stable in plasma, bile and urine up to 90 days of storage at $-60\,^{\circ}C$ and after being subjected to three freeze–thaw cycles. The method was applied to generate the pharmacokinetics of DHP in rats after oral and intravenous administration.

Keywords: Reversed phase chromatography; Dehydropregnenolonehypolipidaemic agents; biomatrix; pharmacokinetics

1. Introduction

Complications of atherosclerosis, such as myocardial infarction, stroke, and peripheral vascular disease still account for half of the deaths in the US. In addition, the quality of life of millions of people is adversely affected by angina and heart failure caused by coronary artery disease [1]. In particular, elevated low density lipoprotein (LDL) levels are responsible for the clinical manifestations of the disease. Disorders of lipoprotein metabolism can lead to hypercholesterolemia, hypertriglyceridemia or both. Furthermore, it has

☆ CDRI Communication No. 6393.

* Corresponding author. Tel.: +91-522-221-2411/12/13/14/15/16/17/18x4277; fax: +91-522-222-3405/3938.
E-mail address: rcgupta@usa.net (R.C. Gupta).

0731-7085/03/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0731-7085(03)00308-X

been shown unequivocally that reducing plasma levels or more authentically decreasing the LDL:high density lipoprotein (HDL) ratio leads to beneficial effect. Diet and exercise often fail to lower total and LDL cholesterol levels adequately, such that pharmacotherapy is needed to achieve National Cholesterol Education Programme (NCEP) guideline goals [2–4].

A decade ago, the choice of lipid lowering drugs was limited to bile acid sequestrant resins, nicotinic acid, gemfibrosil, bezafibrate and clofibrate [5]. The introduction of HMG CoA reductase inhibitors was a major advance in cholesterol lowering therapy. In quest for more potent and safer hypolipidaemic agent, CDRI has developed dehydropregnenolone (DHP) (Fig. 1), an orally acting hypolipidaemic agent. It shows significant hypolipidaemic effect in normal as well as in hyperlipidemic subjects. DHP increase HDL levels, inhibits platelet aggregation and decreases the cholesterol biosynthesis in liver. Chronic toxicity studies indicated that this drug is free from any untoward effects and possess a good therapeutic window (Pratap et al., US patent, 1999, 09.280 448; Nityanand et al., European patent, 1999, 99302556.8). The full-fledged preclinical pharmacokinetic data of DHP is to be generated, which requires the development of a precise and accurate analytical method with optimum LOQ in different biological matrices. The present paper reports the development and validation of an HPLC-UV method for estimation of DHP in various biomatrices and its application to PK studies in rats.

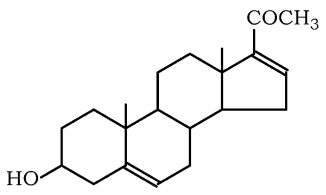

Fig. 1. Chemical structure of DHP.

2. Materials and methods

2.1. Chemicals

DHP (3-β-hydroxy-5, 16-pregnadiene-20-one) (purity $>99\%$) was synthesized at the Medicinal Chemistry Division of Central Drug Research Institute (C.D.R.I), Lucknow. HPLC grade acetonitrile (ACN) and *n*-hexane were procured from J.T. Baker (Philipsburg, USA). Deionized water (DW) was obtained from Milli Q_{PLUS} system (18.2 MΩ cm). Dimethyl sulphoxide (DMSO) was supplied by Spectrochem Pvt. Ltd, (Mumbai, India). All other chemicals were of analytical grade and procured from local sources unless specified. Heparinised blank plasma, bile, urine and feces samples were obtained from drug free male *Sprague Dawley* rats, which were procured from the Laboratory Animal Services Division, C.D.R.I.

2.2. Instruments and equipments

The HPLC system consisted of a pump (LC-10ATvp with SCL 10Avp system controller, Shimadzu, Japan) with flow control valve system (FCV-10ALvp) and a degaser (DGU-14A) to pump the mobile phase. The detection was performed using SPD-10Avp UV–VIS detector set at 248 nm. A Model 7125i syringe loading injector (Rheodyne, USA) with a fixed 100 μl loop was used to inject the samples. Chromatographic separations were performed on Spheri-5, RP-18 column, Applied Biosystems Inc. (100×4.6 mm i.d., 5 μm), coupled with a guard column packed with the same material (30×4.6 mm i.d., 5 μm). The mobile phase was composed of acetonitrile:DW:: 55:45% v/v at a flow rate of 1 ml/min. Mobile phase was degassed for 20 min in a sonicator (Bransonic cleaning Co., USA) before use. Data was analysed using CLASS-VP software (Shimadzu, Japan) running on a Compaq PC. The HPLC system was equilibrated for approximately 30 min at a flow rate of 1 ml/min before the commencement of the analysis. A vortex-mixer (Thermolyne, USA), Model SVC-220H speed vac concentrator (Savant, NY, USA) and Model K130 centrifuge (BHG Hermle) were used for sample

preparation. Plasma, bile and urine samples were stored at −60 °C in Ultra Freeze U41085, Ultra Low Freezer (New Brunswick Scientific, USA). The concentration time data were subjected to both non-compartmental and compartmental approaches using WIN NONLIN software, version 1.5 (SCI consultants, USA).

2.3. Stock and standard solutions

Stock solutions and analytical standards were prepared in HPLC grade acetonitrile. Working stock solutions were made from the mother stock (1 mg/ml) of DHP with ACN. Analytical standards (20–1000 ng/ml) were prepared by appropriate dilution of the working stock solutions. These analytical standards were used to determine HPLC system reproducibility and absolute recovery for DHP from various biomatrices. All solutions were stored at 4 °C for a maximum period of 1 month.

2.4. Calibration curve and quality control samples

Calibration standards (CS) over a range of 20–1000 ng/ml were prepared by spiking blank rat plasma, bile or urine with appropriate working stock solutions, keeping the volume of organic phase spiked ≤2.5% of the biomatrix. Furthermore, CS in blank feces powder were prepared by spiking respective working stocks to 50 mg of feces powder and subsequently drying them at ≤35 °C to remove the organic phase prior to sample processing. Quality control (QC) samples at low (20 ng/ml), medium (200 ng/ml) and high (1000 ng/ml) concentrations were used for the method validation programme.

2.5. Sample cleanup

2.5.1. Plasma, bile and urine samples

A simple and efficient liquid–liquid extraction with 2 × 2.0 ml of *n*-hexane was used to isolate DHP from plasma, bile and urine. Aliquots of blank, spiked or test samples (200 μl) were taken in a 10 ml graduated extraction tube and 2 ml *n*-hexane was added to it, vortex mixed for 60 s and centrifuged at 2000 rpm for 5 min. The organic layer was transferred to another tube by snap freezing the aqueous layer in liquid nitrogen. The aqueous phase was again extracted with 2.0 ml *n*-hexane and the combined organic phases were evaporated to dryness under reduced pressure using Savant speed vac Concentrator. The dry residue was reconstituted in 200 μl ACN, centrifuged and the clear supernatant (∼150 μl) was injected onto the HPLC system.

2.5.2. Feces samples

The sample cleanup method employed for feces was simple extraction with ACN. Fifty milligram dry feces powder (blank, spiked or test) was vortex mixed for 60 s with 3 ml ACN and centrifuged at 2000 rpm for 3 min. The clear supernatant (∼150 μl) was injected on to HPLC without further processing.

3. Method validation

The method was validated in plasma for 5 days in terms of HPLC system reproducibility, sensitivity, specificity, linearity, absolute recovery, accuracy, precision, freeze–thaw cycle stability and long term stability in spiked plasma samples stored at −60 °C. The method was extrapolated to other biomatrices like, bile, urine and feces by performing partial validation in terms of intra assay accuracy and precision [6].

The lower limit of detection (LOD) for DHP was defined as the drug concentration in the plasma, bile, urine and feces after the sample clean up method that corresponds to three times the baseline noise ($S/N > 3$). The limit of quantification (LOQ) was defined as the concentration of the sample that can be quantified with <20% deviation [6].

Linearity for CS ($n = 6$) in triplicates for 5 days was assessed by subjecting the spiked concentrations and the respective peak height to least-square linear regression analysis with and without intercepts, and a weighted least-square regression ($1/x$ or $1/x^2$). A proper calibration model was chosen after examination of residuals and coefficient of correlation in each case [7].

For calculation of absolute recoveries of DHP, spiked QC samples were prepared at low (20 ng/ml), medium (200 ng/ml) and high (1000 ng/ml) concentrations. The samples were processed as mentioned above and the concentration of DHP was determined from the linear regression of the analytical standard curve. The recovery was calculated by comparing the observed concentration with the spiked concentrations.

For the determination of accuracy and precision, CS and QC samples at low, medium and high concentration were analysed in five replicates for 5 different days ($n = 3 \times 5 \times 5 = 75$). Intra- and inter-batch accuracy was determined by calculating the %bias from the theoretical concentration using the following equation:

$$\%\text{Bias} = \frac{\text{Observed concentration} - \text{Nominal concentration}}{\text{Nominal concentration}} 100$$

Inter- and intra-batch precision in terms of relative standard deviation (%R.S.D.) was obtained by subjecting the data to one way analysis of variance (ANOVA).

To assess the freeze–thaw stability of DHP in biological samples, the QC samples at low, medium and high concentrations in rat plasma, bile and urine in triplicate for 4 different days were prepared. One set comprising of triplicate samples at each concentration level was assayed on the day of preparation (no freeze–thaw cycle). The remaining three sets were stored frozen at $-60\,^{\circ}C$ and analysed after one, two and three freeze–thaw cycles and results expressed as percent deviation.

For the long-term stability of DHP in the spiked samples stored at $-60\,^{\circ}C$, the QC samples at low, medium and high concentrations in triplicate for 5 different days were prepared and stored at $-60\,^{\circ}C$. These sets of samples were analysed after 1, 15, 30, 60 and 90 days of storage and their concentrations read from the respective CS curve on that day and results expressed as percent deviation. The long-term stability studies of feces samples stored at room temperature were similarly performed and dry residue stability of the extracted plasma, bile and urine samples were also established.

4. Pharmacokinetic studies and statistical analysis

Young, healthy, male *Sprague Dawley* rats were obtained from the Laboratory Animal Services Division, CDRI. The intravenous dosing formulation of DHP was prepared by accurately weighing 45 mg of DHP and dissolving it in 1.0 ml of DMSO. Rats were dosed at 18 mg/kg and blood samples collected at different time points up to 3.0 h post-dose. Plasma was separated by centrifuging the heparinised blood at 2000 rpm for 10 min and stored at $-60\,^{\circ}C$ till further analysis.

Excretion studies were carried out following oral administration of DHP at 72 mg/kg dose level. After overnight fasting, a suspension of DHP in water using 5% carboxy methyl cellulose (1 ml/kg) was administered using a rat feeding needle to bile cannulated rats. Cannulation of bile duct in rats was performed under light ether anaesthesia and the animals were allowed to recover prior to dosing [8]. Bile, urine and feces samples were collected from these rats maintained in restrainer cages over a period of 24 h-post-dose. The concentration–time data obtained was analysed using WIN NONLIN software and various parameters estimated.

5. Results and discussion

The successful analysis of the analytes in biological fluids using HPLC relies on the optimization of sample preparation, chromatographic separation and post-column detection. Each of these steps were carefully optimized for developing sensitive, selective, and reproducible assay methods for DHP in various biomatrices.

Development of the method was initiated with a Spheri-5, RP-18 column (Applied Biosystems Inc. (100×4.6 mm i.d., 5 μm)) with isocratic elution at 1 ml/min using a mobile phase containing acetonitrile and DW (50:50% v/v). The replacement of DW with phosphate or ammonium acetate buffer (at pH 4, 4.5, 5, 5.5, 6.0) in the mobile phase could not improve the peak characteristics of DHP further. It was observed that an increase in organic modifier content above 60% resulted in improved sensitivity for DHP, but there was interference of

endogenous impurities in the elution zone of the analyte. The workability of the method was also checked on Cyano columns of different makes (Phenomenex and Pierce). This resulted in decrease in sensitivity of DHP along with interference of endogenous impurities. Hence, it was decided to pursue with C18 column (100 × 4.6 mm, 5 μm) and the optimum separation of DHP

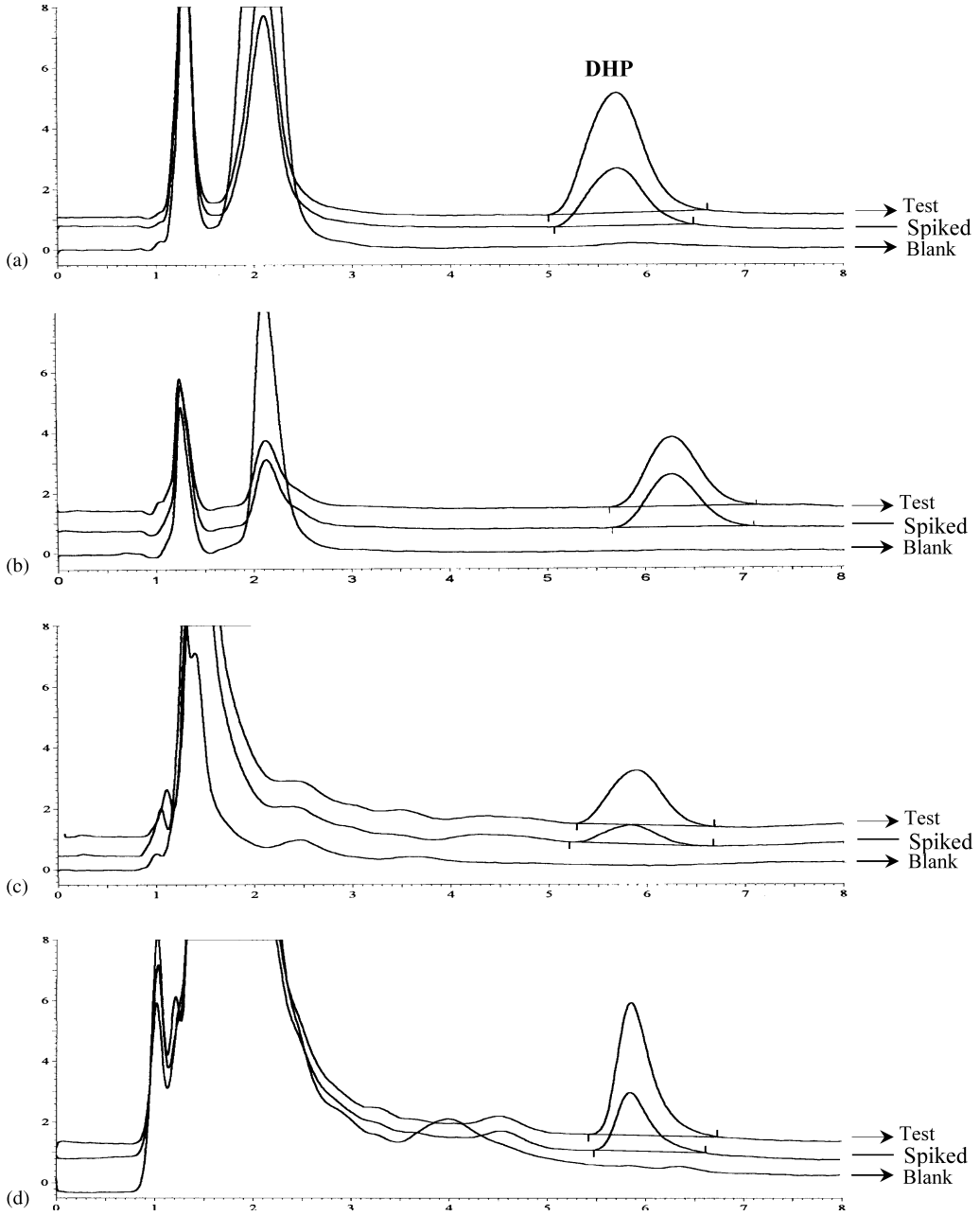

Fig. 2. Representative chromatograms of blank, spiked and test samples of DHP in (a) plasma (b) bile (c) urine and (d) feces sample.

Table 1
Calibration curve equations for DHP in rat plasma, bile, urine and feces

Biomatrix	Equation	Slope	%R.S.D.	Intercept	%R.S.D.
Plasma	$y=mx+c$	0.98	2.89	−54.02	3.97
Bile	$y=mx+c$	2.45	1.89	−26.15	2.13
Urine	$y=mx+c$	11.34	3.20	−193.04	4.63
Feces	$y=mx+c$	7.60	3.37	226.29	4.87

from the endogenous plasma, bile, urine or feces components was achieved using an isocratic elution at 1 ml/min with acetonitrile:DW:: 55:45% v/v as mobile phase. The analyte eluted at 5.7 ± 0.25 min with no endogenous interference.

Various extraction solvents like *n*-hexane, diethyl ether, and ethyl acetate were tried alone and in combinations to assess the recovery of DHP from spiked rat plasma, urine and bile. It was found that double extraction with *n*-hexane yielded clean chromatograms for DHP (absolute recovery $>92\%$) with no endogenous interference.

5.1. HPLC system reproducibility

The HPLC system reproducibility was checked with five replicate injections of each analytical standard. The variations in the peak heights of DHP was maximal at 20 ng/ml (4.3%) and was $<$ 2.0% at all other concentration levels indicating that the system yields reproducible data.

5.2. Selectivity and specificity

Specificity of the assay method is defined as non-interference in the regions of the compound of interest with endogenous substances or drug metabolites for the accurate determination of its concentration. Typical chromatograms of the blank, spiked and test samples in plasma, bile, urine and feces are shown in Fig. 2a–d respectively. The blank chromatograms were free from any endogenous interfering peaks in the region of interest thus confirming the selectivity and specificity of the method. The specificity of the assay methodology was established using eight independent sources of the same matrix.

5.3. Linearity, LOD and LOQ

Calibration curves obtained in rat plasma, bile, urine and feces were found to be linear over a range of 20–1000 ng/ml. An unweighted linear regression scheme ($y=mx+c$) was used to perform standard calibration and the %R.S.D. of slope and intercept are given in Table 1. The correlation coefficients for these biomatrices ($r=0.999\pm0.0008$) indicate linearity over the whole calibration range. LOD and LOQ for DHP were 10 and 20 ng/ml, respectively, in all the biomatrices.

Table 2
Mean absolute recoveries of DHP from spiked rat plasma, bile, urine and feces

Concentration (ng/ml)	%Absolute recovery (Mean±S.D.)			
	Plasma	Bile	Urine	Feces
20	95.8±2.3	93.5±1.9	94.2±4.1	95.6±3.9
200	95.4±3.8	94.8±4.7	96.1±4.8	93.7±2.8
1000	94.2±3.1	92.7±2.5	93.4±4.9	94.0±3.1

Table 3
Accuracy and precision of DHP in spiked rat plasma

Concentration (ng/ml)	Accuracy (%Bias)		Precision (%R.S.D.)	
	Intra day	Inter day	Intra day	Inter day
20	7.1	8.5	9.1	8.8
500	5.6	3.4	6.8	11.2
1000	3.1	1.8	4.7	5.3

5.4. Accuracy and precision

The parameters obtained for accuracy and precision for DHP in plasma are listed in Table 2. The results obtained show that the method was precise and accurate in all the biomatrices with intra- and inter-batch variation within acceptable limits of $\pm 20\%$ at low and $\pm 15\%$ at other concentration levels (Table 3). During partial validation of the method in rat bile, urine and feces, the intra assay accuracy and precision at all the QC levels were less than 8 and 10%, respectively.

5.5. Recovery

Absolute recoveries of DHP at the three concentrations in pentaplet for 5 different days in plasma ranged from 95 to 95.8% with a %CV < 5% at all concentration levels. Similar recoveries were obtained for bile, urine and feces during partial validation in these biomatrices.

5.6. Stability studies

DHP was found to be stable in frozen plasma, bile and urine after three freeze–thaw cycles. The deviation observed after one, two and three freeze–thaw cycles were within $\pm 20\%$ at low and within $\pm 15\%$ at other two concentration levels (Fig. 3). Moreover, there was no indication of degradation of DHP in spiked plasma, urine and bile samples stored at $-60\,^{\circ}C$ till 90 days (Table 4). Long-term stability studies of DHP in dry feces powder at room temperature showed appreciable stability over 2 months. Furthermore DHP in

Table 4
Long-term stability of DHP in rat plasma, bile, urine and feces on storage

Biomatrix	Concentration (ng/ml)	%Deviation observed on different days				
		1	15	30	60	90
Plasma	20	3.6	4.2	6.1	2.5	−1.5
	200	2.4	5.3	7.2	4.3	6.8
	1000	1.0	2.8	3.4	−2.6	8.8
Bile	20	−2.1	−4.5	−6.3	−8.1	−7.8
	200	−1.0	−0.6	4.2	−1.1	2.2
	1000	−1.5	2.8	6.5	−1.3	6.8
Urine	20	5.4	5.2	−8.1	−6.9	−8.4
	200	4.2	4.8	−7.5	−8.0	−7.2
	1000	3.7	6.2	7.8	9	9.2
Feces	20	6.2	−1.2	−3.6	−1.5	−6.8
	200	5.8	−3.5	−1.0	5.0	7.1
	1000	2.4	−4.0	3.2	−4.7	5.9

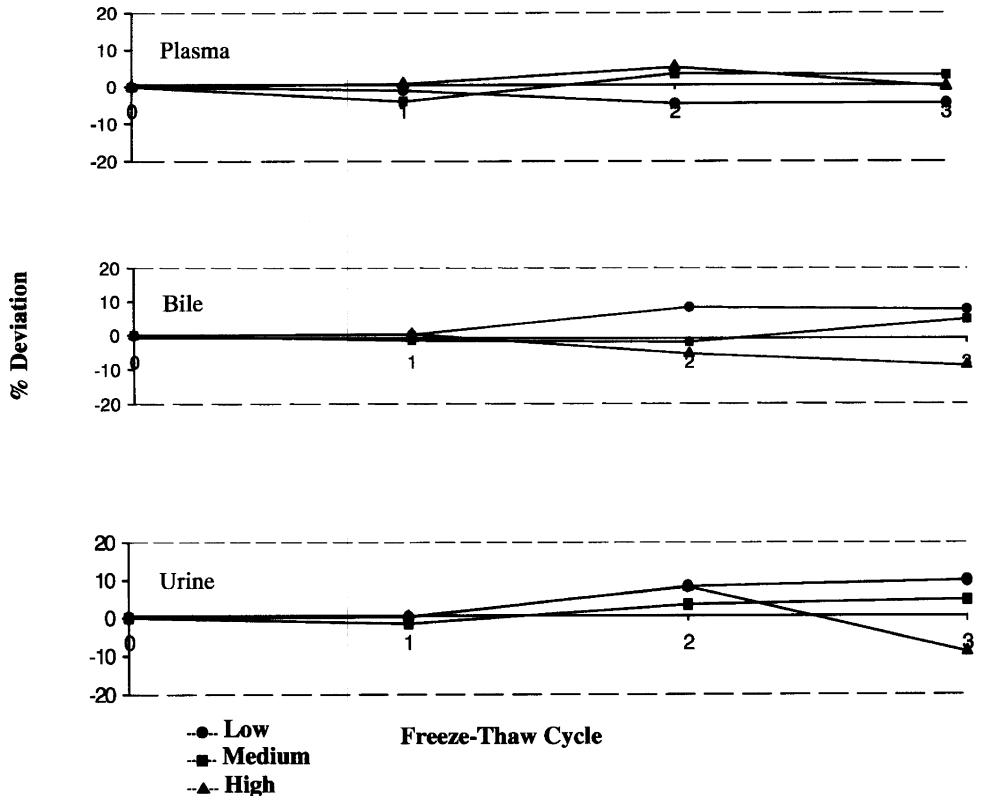

Fig. 3. Freeze–thaw stability of DHP in rat plasma, bile and urine (after one, two and three freeze–thaw cycles) at low, medium and high concentrations.

biomatrix was found to be stable at room temperature for a period of 6 h indicating its bench top stability. The extracted dry residue of plasma, bile and urine was stable for at least 15 days. The reconstituted extracts of plasma, bile and urine samples and the supernatant of feces extract (separated and stored at 4 °C) were stable over a period of 24 h. These parameters establish the re-injection reproducibility of the method.

5.7. Sample dilution

If the concentration of any test sample exceeds 1000 ng/ml, the highest concentration in the calibration curve, a fivefold dilution can be applied. Concentrations of such diluted samples can be measured with appreciable degree of accuracy and precision (%Bias and %R.S.D. $< \pm$ 10).

6. Applicability of the assay to pharmacokinetic studies in rats

The method described here was successfully applied to study the pharmacokinetics of DHP in male *Sprague Dawley* rats. Plasma concentration profile of DHP following a single intravenous dose in rats ($n = 5$) is shown in Fig. 4. DHP was traced up to 3 h and the plasma concentration time profile best fitted to a two compartment open model with first order elimination (correlation $>$ 0.997). The elimination half-life ($t_{1/2}\beta$) was 25.7 min in which β is the elimination rate constant for the terminal exponential phase, and mean resi-

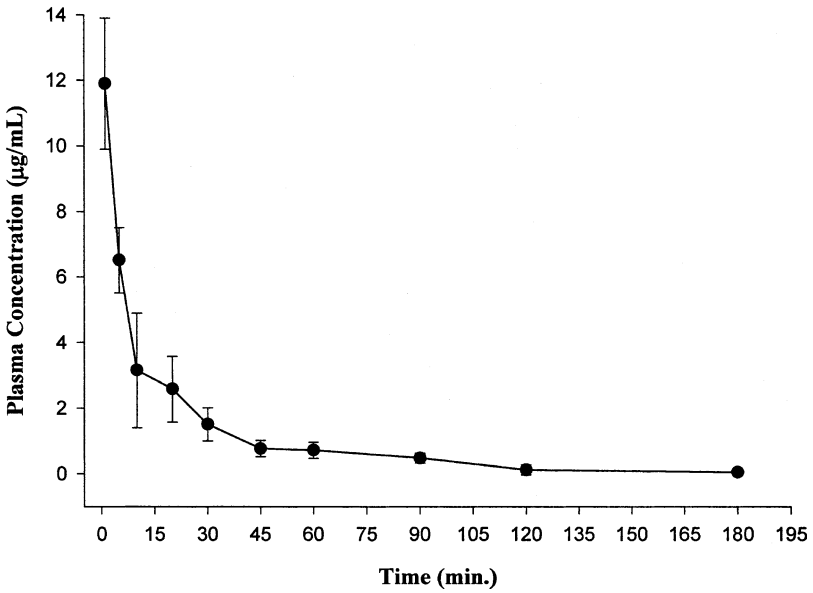

Fig. 4. DHP concentration in plasma following an intravenous dose of 18 mg/kg in rats ($n = 5$).

dence time was estimated to be 28.66 min. Volume of distribution (Vd) and total clearance (Cl) were 1.24 l and 103 ml/min/kg, respectively.

The assay methods developed in bile, urine and feces were used for carrying out the excretion studies following oral administration. In line with other drugs of the same class, the urinary and biliary excretion of DHP were negligible while a significant portion of the orally administered dose (up to 30%) was excreted unchanged in feces.

7. Conclusions

HPLC-UV methods for estimation of DHP in various biomatrices were developed and validated. These methods were found to be specific, accurate and precise. The calibration curves were linear over the concentration range employed (20–1000 ng/ml) and the established sample dilution procedure facilitated the analysis of test samples which were outside the calibration range. There were no stability problems for DHP during storage and sample processing hence fulfilling the criteria for bioanalytical methods. This method was successfully applied to study the pharmacokinetics of DHP in rats following single oral and intravenous administration.

Acknowledgements

The authors are thankful to Director, CDRI for his constant encouragement and support. We also acknowledge Council of Scientific and Industrial Research (CSIR) for providing research fellowships.

References

[1] J.G. Hardman, L.E. Limbird, P.B. Molinoff, R.W. Ruddon, A.G. Gilman, Goodman and Gilmans, The Pharmacological Basis of Therapeutics, ninth ed., McGraw-Hill Companies Inc, New York, 1996, pp. 875–896.
[2] P.H. Chong, J.D. Seeger, C. Franklin, Am. J. Med. 111 (2001) 390–400.
[3] R.H. Knopp, N. Engl. J. Med. 341 (1999) 498–511.
[4] Expert panel on detection, evaluation, and treatment of high blood cholesterol in adults. Summary of the Second Report of the National Cholesterol Education Programme (NCEP), J. Am. Med. Assoc. 269 (1993) 3015–3023.
[5] D. Bhatnagar, Pharmacol. Ther. 79 (1998) 205–230.

[6] V.P. Shah, K.K. Midha, J.W.A. Findlay, H.M. Hill, J.D. Hulse, I.J. McGilveray, G. McKay, K.J. Miller, R.N. Patnaik, M.L. Powel, A. Tonelli, C.T. Viswanathan, A. Yacobi, Pharm. Res. 17 (2000) 1551–1557.
[7] N.V. Nagaraj, J.K. Paliwal, R.C. Gupta, J. Pharm. Biomed. Anal. 20 (1999) 433–438.
[8] H.B. Waynforth, Experimental and Surgical Technique in the Rat, Academic Press, London, 1980, pp. 127–130.