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A sensitive and selective HPLC/ESI-MS/MS assay for the simultaneous quantification of 16-dehydropregnenolone and its major metabolites in rabbit plasma☆

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Abstract

A sensitive, selective and rapid liquid chromatographic/electrospray ionization tandem mass spectrometric assay was developed and validated for the simultaneous quantification of 16-dehydropregnenolone (DHP) and its five metabolites *4,16-pregnadien-3, 20-dione* (M_1), *5-pregnene-3β-ol-20-one* (M_2), *5-pregnene-3β, 20-diol* (M_3), *5-pregnene-3β-ol-16, 17-epoxi-20-one* (M_4) *and 5,16-pregnadien-3β, 11-diol-20-one* (M_5) in rabbit plasma using dexamethasone as internal standard (IS). The analytes were chromatographed on Spheri-5 RP-18 column (5 μm, 100 mm × 4.6 mm i.d.) coupled with guard column using acetonitrile:ammonium acetate buffer (90:10, v/v) as mobile phase at a flow rate of 0.65 ml/min. The quantitation of the analytes was carried out using API 4000 LC-MS–MS system in the multiple reaction monitoring (MRM) mode. The method was validated in terms of linearity, specificity, sensitivity, recovery, accuracy, precision (intra- and inter-assay variation), freeze-thaw, long-term, auto injector and dry residue stability. Linearity in plasma was observed over a concentration range of 1.56–400 ng/ml with a limit of detection (LOD) of 0.78 ng/ml for all analytes except M_3 and M_5 where linearity was over the 3.13–400 ng/ml with LOD of 1.56 ng/ml. The absolute recoveries from plasma were consistent and reproducible over the linearity range for all analytes. The intra- and inter-day accuracy and precision method were within the acceptable limits and the analytes were stable after three freeze-thaw cycles and their dry residues were stable at −60 °C for 15 days. The method was successfully applied to determine concentrations of DHP and its putative metabolites in plasma during a pilot pharmacokinetic study in rabbits.

Keywords: LC-MS–MS; Hypolipidaemic agent; 16-Dehydropregnenolone; Rabbit plasma; MRM

1. Introduction

Hypolipidaemic drugs have attracted considerable attention because of their potential to prevent cardiovascular diseases such as myocardial infarction, stroke and peripheral vascular disease by retarding the progression of atherosclerosis in hyperlipoproteinemic individuals. Epidemiological studies and large scale clinical trials with statin class of cholesterol-lowering drugs have conclusively shown an association between reduced levels of low density lipoprotein cholesterol (LDL-C) and decreased morbidity and mortality from coronary heart disease (CHD) [1–3]. But despite these demonstrated benefits of lowering LDL-C levels many patients receiving cholesterol lowering therapies fail to reach the LDL-C levels recommended by current guidelines [4]. The need or more effective cholesterol lowering has encouraged the use of higher doses of established statins, and also prompted efforts to develop more potent drugs with novel mode of action that can be used to achieve greater reductions in LDL-C levels in a broad spectrum of patients. DHP (Fig. 1), a oral hypolipidaemic agent developed by Central Drug Research Institute (CDRI), Lucknow, shows significant hypolipidaemic effect in normal as well as in hyperlipidaemic subjects. DHP increases HDL levels, inhibits platelet aggregation and decreases the cholesterol biosynthesis in liver. Chronic toxicity studies indicted that this drug is free from any untoward effect and possess a good therapeutic window (Pratap et al., US patent, 1999, 09.280448; Nityanand et al., European patent, 1999, 99302556.8). Preliminary excretion and in vitro metabolic studies performed earlier in our laboratory indicated lower metabolic stability of DHP.

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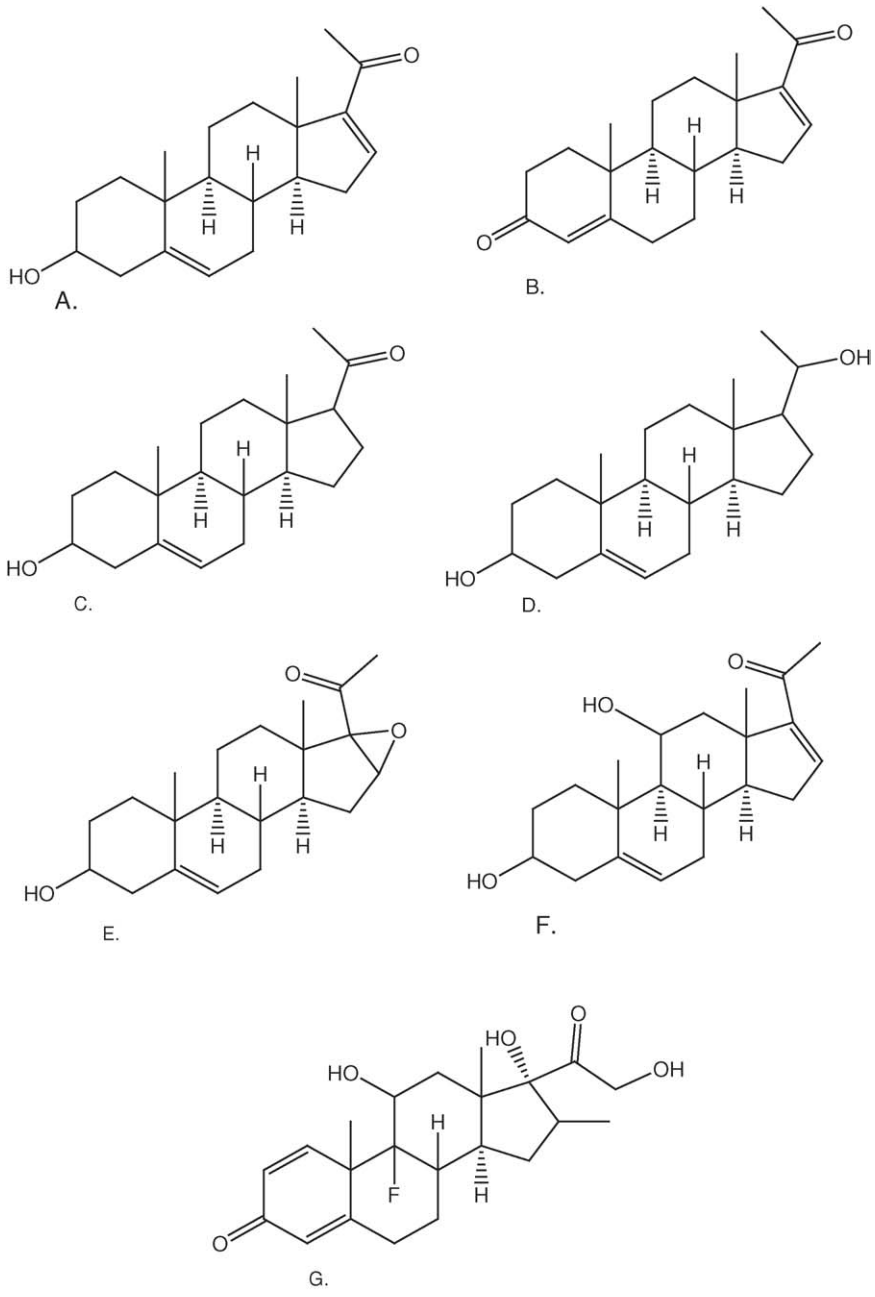

Fig. 1. Chemical structure of (A) DHP (B) M_1 (C) M_2 (D) M_3 (E) M_4 (F) M_5 and (G) IS.

Metabolites of DHP (M_1, M_2, M_4) were then tentatively identified from these studies and two more putative metabolites (M_3 and M_5) were considered while developing the bioanalytical method for pharmacokinetic (PK) evaluation (Fig. 1).

An HPLC-UV method for determination of DHP was reported in rat biological matrix with 20 ng/ml as lowest limit of quantitation (LLOQ) [5]. However, the sensitivity of this assay was found to be inadequate for PK profiling of DHP by conventional routes of administration. Therefore, it was deemed necessary to develop a more sensitive and selective assay method for simultaneous quantitative estimation of DHP and its metabolites in biological fluids for meaningful preclinical pharmacokinetic evaluation to support the development of DHP as a candidate hypolipidaemic drug.

Analysis of steroid most often involves radioimmunoassay (RIA) [6–10], GC–MS [11–15]. RIA suffers from relatively poor specificity due to cross-reactivity of the antibodies [6–8]. Majority of analytical method for endogenous as well as exogenous steroids uses GC–MS which allows the identification of these steroids and metabolites with low detection limits, however requiring extensive sample clean up as well as multistep derivatization procedures.

The advent of atmospheric pressure ionization (API) source is an important breakthrough, and the use of LC-MS–MS with an ESI (electro spray ionization)/APCI (atmospheric pressure chemical ionization) interface is a sensitive and selective technique that is currently considered as the method of choice for pharmacokinetic studies [16–21]. Reversed

phase-HPLC separation in combination with tandem mass spectrometry with APCI (LC-APCI-MS–MS) have been used to monitored common biologically active natural and synthetic steroids [22]. However, there is lack of literature on the analysis of endogenous as well as exogenous DHP using LC-MS–MS.

This paper present, for the first time, the development and validation of highly sensitive, selective and specific LC-MS–MS method in multiple reaction monitoring (MRM) mode for the simultaneous quantification of DHP and its metabolites in rabbit plasma using dexamethasone as internal standard (IS). The method was successfully applied to detect levels of DHP and its metabolites after oral administration in rabbits.

2. Experimental

2.1. Chemicals and reagents

DHP (*5,16-pregnadien-3β-ol-20-one*) (purity > 99%) was synthesized at the Medicinal and Process Chemistry Division of CDRI, Lucknow, India. Reference standards of *4,16-pregnadien-3, 20-dione* (*M_1*), *5-pregnene-3β-ol-20-one* (*M_2*), *5-pregnene-3β, 20-diol* (*M_3*), *5-pregnene-3β-ol-16, 17-epoxi-20-one* (*M_4*) and *5,16-pregnadien-3β, 11-diol-20-one* (*M_5*) (purity > 99%) were synthesized by one of the authors (S.K. Singh) at Pharmacokinetics and Metabolism Division, CDRI. Dexamethasone (purity > 99%) was purchased from HiMedia Laboratories Pvt. Ltd, Mumbai, India. HPLC grade acetonitrile and isopropyl alcohol (IPA) were procured from Thomas Bakers (Chemicals) Limited, Mumbai, India. *n*-Hexane (HPLC-grade) was purchased from Spectrochem Pvt. Ltd., Mumbai, India. Ammonium acetate (GR grade) and glacial acetic acid (AR grade) were purchased from E Merck (India) Ltd., Mumbai, India. Heparin sodium injection i.p. (1000 IU/ml) was procured from Biologicals E. Limited, Hyderabad, India. Ultra pure water (18.2 MΩ/cm) was obtained from a Milli-Q $_{PLUS}$ PF water purification system. Drug-free heparinised plasma was obtained from different young, healthy male NZ rabbits housed in the Laboratory Animal Services Division of the institute. Plasma samples were stored in glass tube at −60 °C till further use. The studies were carried out as per the guideline of the local ethical committee on animal experimentation.

2.2. Chromatographic conditions

A Perkin-Elmer Series 200 HPLC system (Perkin-Elmer, USA) consisting of flow control valve, vacuum degasser, pump and autosampler was used to deliver mobile phase [solvent A: acetonitrile and solvent B: ammonium acetate buffer, 10 mM in the ratio 90:10%, v/v] at a flow rate of 0.65 ml/min. The mobile phase was degassed for 20 min in an ultrasonic bath (Bransonic Cleaning Equipment Company, USA) prior to use. Chromatographic separations were achieved on Spheri-5 RP-18 column (5 μm, 100 mm × 4.6 mm i.d., Pierce Chemical Company, Rockford, USA) preceded with guard column packed with the same material (30 mm × 4.6 mm i.d., 5 μm). The samples (20 μl) were injected through autoinjector on to the LC-MS–MS system.

Table 1
Optimized MRM condition for DHP, its metabolites and IS

Analytes	Parent ion	Product ion	Declustering potential (V)	Collision energy (eV)
DHP	315.1$[M+H]^+$	137.5	90	41
M_1	313.0$[M+H]^+$	97.2	75	30
M_2	334.3$[M+NH_4]^+$	281.3	45	24
M_3	336.3$[M+NH_4]^+$	283.4	40	19
M_4	348.4$[M+NH_4]^+$	271.5	40	15
M_5	348.4$[M+NH_4]^+$	157.3	40	30
IS	393.2$[M+H]^+$	171.1	80	40

2.3. Mass spectrometric conditions

API-4000 LC-MS–MS (Applied Biosystems/MDS SCIEX, Toronto, Canada) mass spectrometer was operated with standard ESI source coupled with a LC separation system. Analyst 1.4.2 software (Applied Biosystems/MDS SCIEX, Toronto, Canada) was used for the control of equipment, data acquisition and analysis. For optimization of MS parameters, approximately equimolar solutions of each analyte were prepared in acetonitrile:ammonium acetate buffer (50:50%, v/v). Zero air was used as nebulizing gas (GS 1, 25 psi) and nitrogen as curtain gas (20 psi). Declustering potential (DP) was optimized while ion spray voltage, nebulizing and curtain gas conditions were used in default mode. The dwell time and mass width were set at 0.2 s and ±10 amu and MS scan was performed in both positive and negative ion modes.

The product ion spectrum (MS–MS) was generated at optimized DPs to identify the prominent product ions of the analytes using nitrogen as the collision gas. Collision energies (CE) optimization for the precursor to product ions transition was obtained by CE ramping by direct infusion. The established MRM operating conditions are summarized in Table 1.

2.4. Standard and working solutions

Standard stock solutions (1000 μg/ml) of DHP, metabolites (M_1, M_2, M_3, M_4 and M_5) and IS were prepared by accurately weighing 10 mg of each analytes in 10 ml volumetric flask and volume was made up with acetonitrile. A mixed working stock solution (MWS, 10 μg/ml) of DHP and five metabolites were prepared in acetonitrile:ammonium acetate buffer (90:10%, v/v) and working stock for IS (5 μg/ml) was prepared in acetonitrile.

Analytical standards were prepared from MWS by diluting it with reconstituting solution (acetonitrile:ammonium acetate, 90:10%, v/v) over concentration range of 1.56–400 ng/ml for all the analytes by serial dilution method. IS was spiked to each analytical standard to achieve a concentration of 500 ng/ml. All stock and mixed working stock solutions were prepared prior to start of validation and stored at 4 °C. These solutions were found to be stable and used for the complete method validation programme.

2.5. Calibration standards and quality control samples

Calibration standards were prepared from MWS in normal rabbit plasma over concentration range of 1.56–400 ng/ml for DHP and metabolites by the serial dilution. IS (10 μl) was added to the plasma samples (0.1 ml) before extraction. Quality control (QC) samples at three different concentration levels 3.13, 50, 200 ng/ml as low, medium and high, respectively, except M_3 and M_5 for which 6.25 ng/ml was considered as low were prepared in five replicate each day and were used to assess accuracy and precision of the assay method. The calibration standards and quality control samples were prepared fresh on each day of validation.

2.6. Sample cleanup

Sample preparation involved a simple two-step liquid-liquid extraction (2 × 2 ml) with distilled *n*-hexane:IPA::98:2%, v/v. The extraction solution was added to 0.1 ml aliquots of blank/spiked plasma or test samples and vortex mixed (Type 37600 mixer, Thermolyne, USA) for 60 s. Centrifuged (2000 rpm for 5 min) and the upper organic layer was transferred to another set of clean tubes by snap freezing the lower aqueous layer with liquid nitrogen. The same processes were repeated in second step. Combined organic phase was evaporated under reduced pressure in Savant Speed Vac (USA) at 40 °C. The dry residue was reconstituted in 0.1 ml reconstituting solution. The samples (20 μl) were injected onto the LC-MS–MS system.

2.7. Method validation

The method was validated in terms of linearity, specificity, LOD and LLOQ, recovery, accuracy, precision, freeze-thaw, long-term, auto injector and dry residue stability [23]. The accuracy and precision determination were carried out in five replicates for 5 days at low, medium and high concentration levels.

2.7.1. Linearity

Linearity for calibration standards in triplicates was assessed by subjecting the spiked concentrations and the respective peak areas 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 [24].

2.7.2. Specificity and selectivity

Six individual batches of control drug-free rabbit plasma samples were analyzed to ensure that no endogenous interference with the mass transitions chosen for DHP, its five metabolites and IS. To check the selectivity of the method between the analytes, individual standard solutions at their upper limit of quantification (ULOQ) were separately injected and analyzed using current MRM method. For e.g. an ULOQ solution of DHP should not produce peak >20% of LLOQ of any other transition of its metabolites, which was chosen as standard for selectivity. The same was done individually for all analytes to establish selectivity.

2.7.3. LOD and LLOQ

The LOD for DHP and five metabolites were defined as the drug concentration in the plasma after the sample clean up method that corresponds to three times the baseline noise (S/N ≥ 3). The LLOQ was defined as the concentration of the sample that can be quantified with <20% deviation (S/N ≥ 10).

2.7.4. Ion suppression and recovery

Control drug free plasma was extracted with *n*-hexane:IPA::98:2%, v/v, and evaporated to dryness. Dry extracts were dissolved using analytes and IS standard solutions that represent 100% recovery. Ion suppression was determined by comparing the analytical response of these samples with that of standard solutions. Recovery was determined by comparing the response of processed quality control samples with the analytical response of blank samples reconstituted with standard solutions. These experiments were performed at three-concentration levels (low, medium and high) in triplicate. Overall recovery corresponds to the net response after subtraction of the ion suppression and the signal loss due to the extraction.

2.7.5. Accuracy and precision

For the validation of the assay, QC samples were prepared with three concentrations levels of low, medium and high. Five replicate of each QC sample were analyzed together with a set of calibration standards. The accuracy of each sample preparation was determined by injection of calibration samples and three QC samples in five replicate for 5 days. The precision was determined by one-way ANOVA as within and between % R.S.D. [25]. The accuracy was expressed as % bias:

$$\%\,\text{Bias} = \frac{(\text{observed concentration} - \text{nominal concentration})}{\text{nominal concentration}} \times 100$$

2.7.6. Stability studies

The stability of DHP and its metabolites was investigated in the stock and working solutions, in plasma during storage, during processing, after three freeze-thaw cycles and in the final extract. Analytes were considered stable in the biological matrix when 80–120% of the initial concentration was found in case of low and 85–115% at other concentrations. Stability in stock and working solutions was also investigated for the internal standards. Analytes and internal standards were considered stable in the stock and working solutions when 95–105% of the original concentration was recovered.

2.7.6.1. Freeze-thaw (f-t) stability and long-term stability. QC samples at low and high concentration in pentaplet (six sets) were prepared. One set of samples at each concentration level was analyzed immediately after spiking which served as the reference concentration while other five sets were stored at −60 °C. Three sets were analyzed after 1, 2 and 3 f-t cycles in different run. Thawing was achieved by keeping the stored samples undisturbed at ambient temperature for 30 min. The remaining two sets of QC sample were analyzed after 15 and 30 days without any freeze-thaw cycles. The change in concentration during the f-t cycles and long-term storage in rabbit plasma were

determined by comparing the observed concentrations with the reference concentration and expressed as % deviation.

2.7.6.2. Dry residue stability. QC samples at low and high concentration in pentaplet were processed and one set was stored at −60 °C in glass tubes. One set of samples was analyzed immediately which served as the reference concentration. The other set was analyzed after 15 days. The change in concentration was determined by comparing the concentrations observed after 15 days with the reference concentration and was expressed as % deviation.

2.7.6.3. Auto injector stability. Replicates ($n = 5$) of the spiked samples at low and high concentration in pentaplet were processed and reconstituted at the same time. The reconstituted samples were placed in the auto injector, and one set was injected immediately and the other after 24 h. The percent deviation for these two concentration levels was calculated.

2.8. Application to pharmacokinetic study

The method was successfully applied to determine the PK parameters of DHP and metabolites following oral administration at 40 mg/kg dose in male rabbits. Aqueous suspension of DHP with 0.5%, w/v, carboxy methylcellulose was used in the present study. Blood samples were collected at 0.25, 0.5, 0.75, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0, 18.0, 24.0, 36.0 and 48.0 h post dose from the marginal ear vein, plasma separated and analyzed using the present method.

3. Results and discussion

3.1. Mass spectrometry

A mass spectra of DHP and metabolites in acetonitrile–water (50:50%, v/v) was recorded in the positive ion mode with scan range from 200 to 500 amu. Analyte concentrations were of the order of 1 μg/ml. The protonated analytes, $[M + H]^+$ and the ions resulting from the progressive loss of water ($[M + H]^+$-H_2O, $[M + H]^+$-$2H_2O$) were evident in these spectra. Mass spectra of compounds like M_1 which contains α, β unsaturated keto group showed prominent $[M + H]^+$ while DHP, M_2, M_3, M_4 and M_5 containing both keto and a hydroxy groups gave ions (m/z) corresponding to $[M + H]^+$ and $[M + H]^+$-H_2O/$2H_2O$.

The stability of the protonated molecular ions in gas phase can significantly affect the sensitivity of various steroids. Unstable compounds or ions either thermally decompose or produce large number of fragments causing a decrease in signal intensity. For ketosteroids, protonation usually predominantly occurs on ketone group. Furthermore, conjugation of this group with C=C stabilizes the protonated molecule significantly. For compounds with only hydroxy group(s), protonation usually results in facile loss of water. The combination of low proton affinities and multiple ion formation by the loss of water molecules resulted in relatively low signals observed for these classes of compounds [26].

3.2. Optimization of LC-MS–MS condition

For the bioanalysis of multiple analytes, mass spectrometry has become irreplaceable, especially where compounds such as DHP and its structurally similar metabolites are concerned. A chromatographic baseline separation of these compounds would result in longer run times. LC-MS–MS offers unmatched selectivity and specificity thus, the need for chromatographic resolution of all the analytes can be dispensed with, while using tandem mass spectrometry except when one deals with isomers with same precursor to product ion transitions.

In positive ion mode DHP, M_1, M_2, M_3, M_4, M_5 and IS gave protonated, sodium and potassium adducts with varying signal intensity. The division of signal between sodium, potassium and protonated ions resulted in compromised sensitivity. Hence, possibility of formation of intense protonated or ammonium adduct was explored, using ammonium acetate buffer in combination with acetonitrile [27–29]. Sodium adducts are not normally employed as parent ions in MRM mode due to their high stability and erratic fragmentation pattern. Therefore, $[M + H]^+$ and/or $[M + NH_4]^+$ ions, which on fragmentation gave prominent and stable product ions were selected for further developmental work. DP optimization, for the protonated $[M + H]^+$/ammonium adducts $[M + NH_4]^+$ of DHP, M_1, M_2, M_3, M_4, M_5 and IS were carried out by constant infusion (Harvard Apparatus, Pump 11, Holliston, MA, USA). The influence of buffer molarity, pH and types of organic modifier on signal intensity was also studied. Based on the signal intensity of respective protonated and ammonium adducts ions, 10 mM ammonium acetate-acetic acid (pH ∼ 6) and acetonitrile, as the organic phase, were selected for further studies.

Direct infusion MS–MS analyses were carried out to obtain the product ion spectra at different CE. The resulting product ion mass spectra are given in Fig. 2. In brief MS–MS spectra of all analytes yielded fragment ion characteristic of steroids with different intensity depending on position of double bond and presence of hydroxy group [26,30]. Utilizing this information, two or three intense product ions were selected, their collision energies optimized and included in MRM method. Different transitions were compared on the basis of S/N ratio with on-column injection analysis. It was observed that some MRM transition even with most intense product ion gave low S/N than other less intense product ion due to higher inherent noise in these transitions. The transitions selected were m/z 315.1 > 137.5, 313 > 97.2, 334.3 > 281.3, 336.3 > 283.4, 348.4 > 271.5, 157.3, 348.4 > 157.3 and 393.2 > 171.1 for DHP, M_1, M_2, M_3, M_4, M_5 and IS, respectively. In case of M_4 two transitions were considered to increase its sensitivity. Nebulization gas (GS1), turbo gas (GS2), curtain gas and temperature were set to 30, 40, and 15 psi and 100 °C, respectively (1 psi = 6894.76 Pa). Dexamethasone was selected as IS because of similarity in structure and fragmentation pattern, therefore providing better linearity, accuracy and precision. The corresponding final MRM conditions for DHP, metabolites and IS are summarized in Table 1. The acetonitrile content in the mobile phase was found to be critical for the elution of the analytes. So a balance between signal intensities and chromatographic resolution was achieved

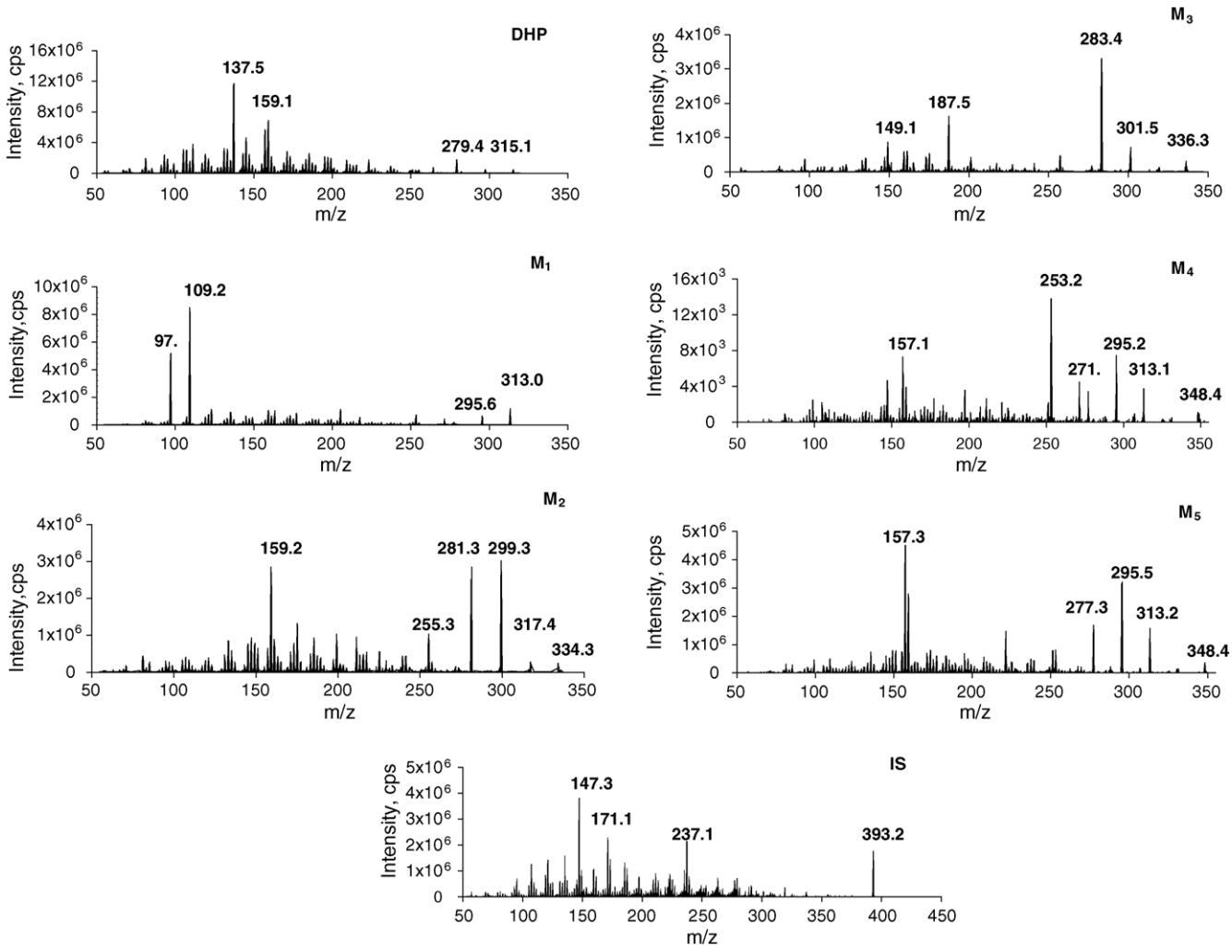

Fig. 2. Product ion spectra of DHP, M_1, M_2, M_3, M_4, M_5 and IS.

with 90% organic content in the mobile phase and the retention times of DHP, M_1, M_2, M_3, M_4, M_5 and IS were found to be 3.6, 3.3, 3.7, 3.8, 3.0, 2.5 and 2.2 min, respectively, at a flow rate of 0.65 ml/min, thus increasing the throughput in simultaneous estimation (Fig. 3).

3.3. Sample cleanup

The next step was to develop an efficient sample clean up devoid of matrix suppression and interference from endogenous plasma components for estimation of the analytes in rabbit plasma. Protein precipitation using acetonitrile could not be followed due to severe matrix suppression (~70%). Liquid–liquid extraction using hexane alone and with different combinations of hexane and ethyl acetate (90–10%, v/v), *n*-hexane and IPA (2–5%, v/v) was tried to get maximum recovery with minimum ion suppression in elution region. Extraction using *n*-hexane and its combination with ethyl acetate also failed to get consistent recoveries with minimum matrix suppression in elution region. Combination of *n*-hexane and IPA was found suitable for recovery of all analytes as well as IS. Though increase in IPA significantly increase the recoveries of M_5 and IS, the recoveries of M_5 and IS were compromised to 45 and 50%, respectively, due to endogenous interference in the elution region of other analytes as well. The final sample clean up was thus optimized to *n*-hexane:IPA::98:2%, v/v, 2 × 2 ml with high selectivity for all analytes and no matrix suppression.

3.4. Assay validation

3.4.1. Linearity and calibration standards

The peak area ratios of analytes to IS were linear over a concentration range of 1.56–400 ng/ml for DHP, M_1, M_2, M_4 and 3.13–400 ng/ml for M_3 and M_5, respectively (Table 2). The calibration model was selected based on the analysis of the data by linear regression with and without intercepts ($y = mx + c$ and $y = mx$) and weighting factors ($1/x$, $1/x^2$ and $1/\log x$). The best fit for the calibration curve could be achieved by a linear equation of $y = mx + c$ and a $1/x^2$ weighting factor for all the analytes with minimum of residuals and regression coefficient >0.998.

3.4.2. Specificity and selectivity

Chromatograms of six batches of control drug-free plasma contained no co-eluting peaks >20% of analytes area at LLOQ

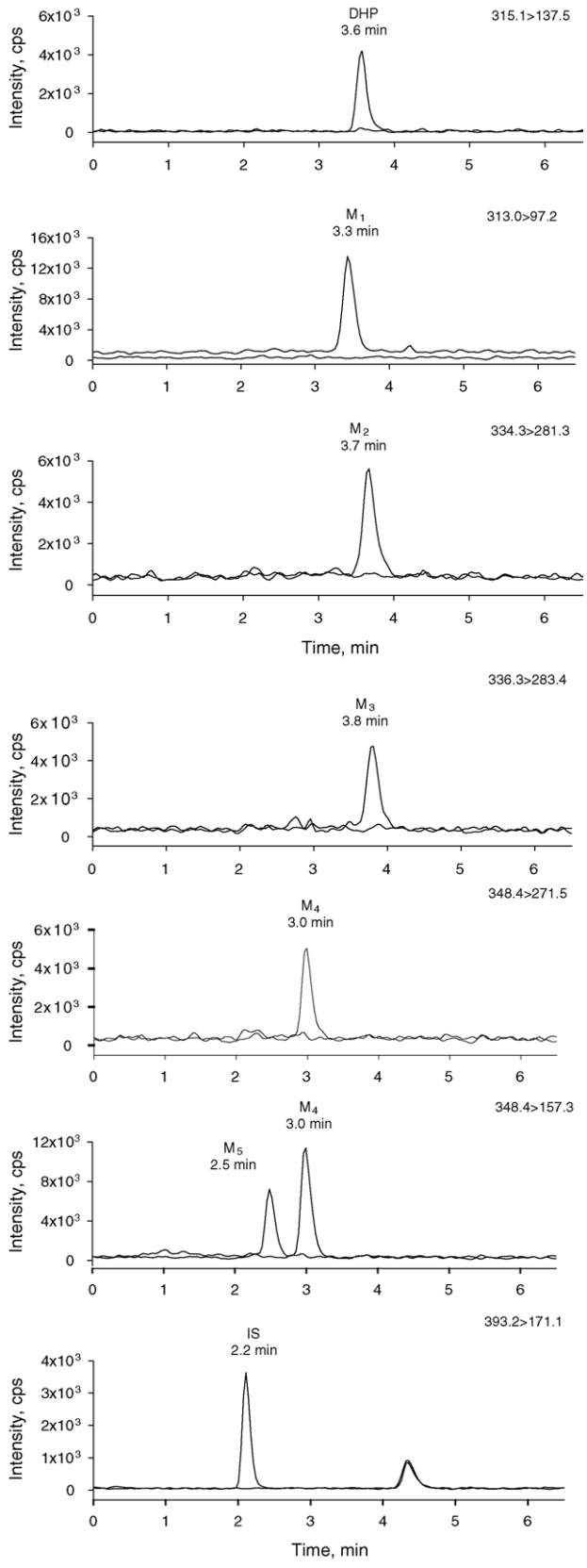

Fig. 3. Representative chromatograms of DHP, M_1, M_2, M_3, M_4, M_5 and IS in fortified blank rabbit plasma overlaid with extracted blank rabbit plasma.

Table 2
Assay linearity of the method

Analyte	Slope	Intercept	Correlation coefficient (R^2)
DHP	0.0131 ± 0.0009	0.0054 ± 0.0051	0.999 ± 0.0028
M_1	0.2910 ± 0.0253	−0.0763 ± 0.0737	0.999 ± 0.0032
M_2	0.0781 ± 0.1201	0.0267 ± 0.0188	0.998 ± 0.0021
M_3	0.0108 ± 0.0039	0.0024 ± 0.0075	0.998 ± 0.0016
M_4	0.0830 ± 0.0163	−0.0140 ± 0.014	0.998 ± 0.0052
M_5	0.0353 ± 0.0577	−0.0008 ± 0.0231	0.999 ± 0.0034

Values are mean ± S.D., $n = 3$.

level and no co-eluting peaks >5% of the area of IS. There was no cross interference between the analytes after subjecting individual analytes to the procedure discussed in Section 2.7.2. Representative chromatograms of extracted blank plasma, blank plasma fortified with DHP, its five metabolites and IS are shown in Fig. 3. The retention times of all the analytes and IS showed less variability with a relative standard deviation (R.S.D.) well within the acceptable limit of 5% [31].

3.4.3. LOD and LLOQ

The LOD demonstrated that all the analytes gave an S/N of ≥3 for 0.78 ng/ml except for M_3 and M_5 for which LOD was 1.56 ng/ml. The LLOQ, the lowest concentration in the standard curve which can be measured with acceptable accuracy and precision for DHP, M_1, M_2, M_4 from normal rabbit plasma was established as 1.56 and 3.13 ng/ml for M_3 and M_5. The LLOQ was established with three QC samples independent of the standard curve. There was 15 times increase in sensitivity for DHP, with a decrease in injection volumes by five-fold leading to higher sensitivity and throughput from the earlier reported HPLC-UV method [5].

3.4.4. Ion suppression and recovery

The possibility of matrix effects on ionization was explored by comparing the responses obtained from blank plasma extracts spiked with analytes and IS reference solutions with that of reference solutions of same concentrations in reconstituting solution. This study was carried out at three concentration levels. There was no significant difference between the peak areas of reference solutions and reference solution spiked in extracted plasma.

The average absolute recoveries for DHP and its metabolites at three different concentrations are shown in Table 3.

3.4.5. Accuracy and precision

Accuracy and precision (intra- and inter-day) were calculated at three different concentration levels of low, medium and high QC samples for all analytes on 5 days are presented in Table 4. The results showed that the analytical method is accurate, as the bias is within the acceptance limits of ±20% of the theoretical value at LLOQ and ±15% at all other concentration levels. The precision around the mean value was never greater than 15% at any of the concentrations studied.

Table 3
Absolute recoveries of DHP, M_1, M_2, M_3, M_4 and M_5 from rabbit plasma

Concentration (ng/ml)	% absolute recovery (mean ± S.D., $n = 5$)					
	DHP	M_1	M_2	M_3	M_4	M_5
3.13	96.4 ± 3.79	90.72 ± 5.11	91.56 ± 4.41	–	94.64 ± 5.79	–
6.25	84.86 ± 3.70	85.96 ± 4.61	90.4 ± 5.08	98.2 ± 3.30	88.3 ± 3.54	55.52 ± 0.90
50	87.48 ± 4.05	85.2 ± 4.41	89.86 ± 1.38	95.5 ± 1.56	85.26 ± 5.16	52.34 ± 2.41
200	86.96 ± 3.14	94.56 ± 2.92	92.96 ± 3.48	97.2 ± 0.36	92.3 ± 3.64	56.23 ± 1.28

3.4.6. Stability studies

All analytes and IS were stable in the stock and working solutions for over 24 h at ambient temperature. Furthermore, analytes and IS were stable at least 3 months in the stock solution at 4 °C, since deviations in concentration from reference solution never exceed 5%. The standard containing DHP and its metabolites were found to be stable in plasma for 8 h at room temperature, which encompasses the duration of typical sample handling and processing. Moreover, the analytes were found to be stable after reconstitution in acetonitrile for at least 12 h at 4 °C.

3.4.6.1. Freeze-thaw (f-t) stability and long-term stability. The deviation observed after first, second and third f-t cycles were within ±15% as is evident from the Table 5 at the concentration levels used for DHP and its five metabolites indicating adequate freeze-thaw stability. It should be noted that these variations represent both stability parameters and the inherent inter and intra batch variations. Also, the QC samples stored at −60 °C were analyzed after 15 and 30 days and there were no significant deviations with respect to the immediately analyzed samples (Table 6).

Table 4
Accuracy (% bias) and precision (% R.S.D.) of analytes

Analytes	Concentration	Accuracy (% bias)		Precision (% R.S.D)	
	ng ml^{-1}	Inter-day	Intra-day	Inter-day	Intra-day
DHP	3.13	1.88	4.05	9.73	8.18
	50	−6.51	−3.72	10.73	4.66
	200	6.43	5.41	3.86	5.12
M_1	3.13	−5.08	−4.91	4.20	4.60
	50	0.42	−0.56	9.66	3.80
	200	6.69	6.05	6.34	4.58
M_2	3.13	−1.72	−2.08	9.94	13.23
	50	3.50	4.59	7.33	4.88
	200	12.50	10.27	9.24	3.24
M_3[a]	6.25	2.13	−0.14	7.78	8.02
	50	−4.04	−3.02	9.51	6.60
	200	7.98	1.81	8.62	8.26
M_4	3.13	3.69	5.66	2.70	4.06
	50	1.42	1.63	5.86	4.14
	200	5.38	4.17	6.58	4.13
M_5[a]	6.25	2.41	2.06	1.84	7.65
	50	−3.66	−0.55	5.16	7.62
	200	9.86	9.92	7.53	2.93

[a] For M_3 and M_5, quality control sample at 6.25 ng ml^{-1} was used as low quality control.

Table 5
Freeze-thaw (f-t) stability data for DHP and its metabolites

Analytes	Nominal concentration (ng/ml)	% deviation		
		f-t 1	f-t 2	f-t 3
DHP	3.13	3.88	−0.87	−6.99
	200	2.75	−7.47	2.44
M_1	3.13	−2.80	−1.70	−10.29
	200	3.84	−4.74	4.08
M_2	3.13	−7.65	1.29	−7.73
	200	−0.55	−8.13	0.48
M_3	6.25	3.59	2.84	7.50
	200	1.04	4.67	4.67
M_4	3.13	−5.94	−9.73	−5.22
	200	5.81	−9.34	7.75
M_5	6.25	−1.71	7.94	−0.38
	200	−0.55	10.33	1.09

3.4.6.2. Dry residue stability. The dry residue samples stored at −60 °C after extraction were found to be stable for over 15 days with % deviation ≤10% at all concentration levels.

3.4.6.3. Auto injector stability and re-injection reproducibility. There was no significant difference between the responses of standards at time zero and after 24 h kept at auto injector at 4 °C in terms of % CV (≤5%) for all analytes, indicating sufficient stability in auto injector for completing large set of analysis.

Table 6
Stability data for DHP, M_1, M_2, M_3, M_4 and M_5 at −60 °C

Analytes	Concentration (ng/ml)	% deviation		
		8 h	7 days	30 days
DHP	3.13	3.88	−0.87	−6.99
	200	2.06	5.06	−0.79
M_1	3.13	−2.80	−1.70	−10.29
	200	2.06	8.64	−3.50
M_2	3.13	−7.65	1.29	−7.73
	200	−4.76	−1.44	−4.04
M_3	6.25	3.59	2.84	7.50
	200	1.04	4.67	4.67
M_4	3.13	−5.94	−9.73	−5.22
	200	−8.07	−9.83	−6.82
M_5	6.25	−1.71	7.94	−0.38
	200	−0.55	10.33	1.09

The re-injection reproducibility in all cases established with the percent deviations was less than ±15% at all concentration levels.

3.5. Internal standard

In quantitative MS, internal standards are used for the accurate determination of concentrations. Since the ionization efficiency in ESI is highly dependent on the structure of compounds, internal standards are preferably stable isotopically labeled analogues of the analytes or a compound with a structure closely resembling the parent drug [32–34]. Since a stable isotopically labeled internal standard was not available, dexamethasone was selected as IS. Dexamethasone gave ionization efficiency comparable to that of the analytes, no significant matrix effect and was quite stable as well.

3.6. Application to pharmacokinetic study

The method was applied to determine levels of DHP and its metabolites post oral dosing in rabbit ($n = 1$). Plasma concentration–time profile of DHP and its metabolites after 40 mg/kg oral administration is shown in Fig. 4. The plasma concentration–time profile of M_3 and M_5 could not be generated because of very low levels. The PK parameters of DHP and metabolites (M_1, M_2 and M_4) are given in Table 7. The plasma concentration time profile of DHP and metabolites was best fitted to non compartment model using WinNonlin (WinNonlin, standard edition, ver 1.5, USA). The elimination half life of DHP, M_1, M_2 and M_4 were 5.95, 4.68, 2.68 and 3.26 h, respectively. Thus, the method was found suitable for conducting pharmacokinetic studies.

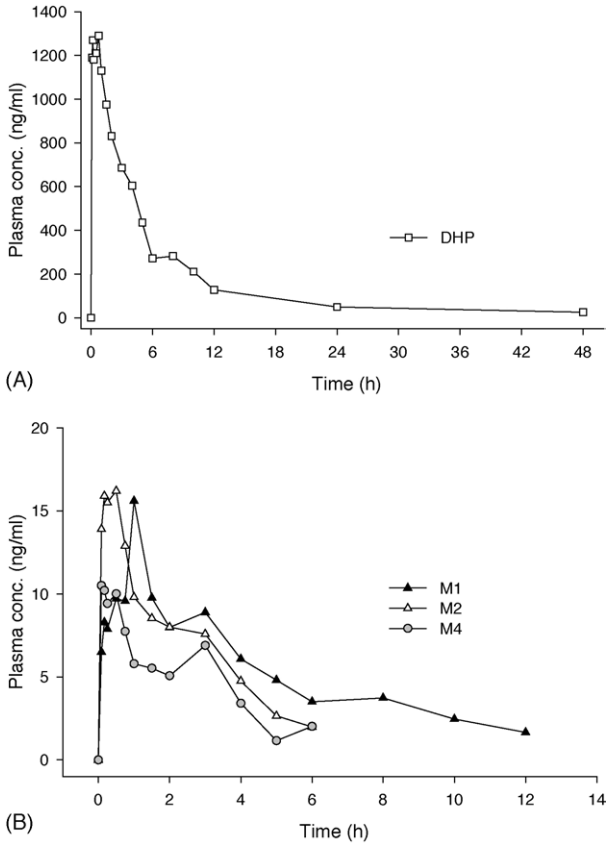

Fig. 4. Plasma concentration–time profile ($n = 1$) of (A) DHP and (B) metabolites (M_1, M_2 and M_4) after 40 mg/kg oral administration in rabbit.

Table 7
PK parameters of DHP after 40 mg/kg oral administration in rabbit

PK parameter	Dose: 40 mg/kg oral			
	DHP	M_1	M_2	M_4
C_{max} (ng/ml)	1290	15.6	16.2	9.42
t_{max} (h)	0.75	1	0.5	0.25
$AUC_{0\text{-inf}}$ (ng h/ml)	7992.75	74.36	55.92	39.98
$t_{1/2}$ (h)	5.95	4.68	2.68	3.26
Cl/F (L/h)	4.91	–[a]	–[a]	–[a]
V_d/F (L)	42.95	–[a]	–[a]	–[a]
MRT (h)	9.98	6.38	3.88	4.30

The pilot study was carried out in one NZ rabbit. M_3 and M_5 levels are too low to carryout PK analysis.
[a] Not applicable.

4. Conclusion

An LC-MS–MS bioanalytical method for simultaneous determination of DHP and its five metabolites were developed and validated in rabbit plasma. This method has significant advantages over previously reported, in terms of sensitivity, selectivity and shorter run time (6.5 min). The established LLOQ of 1.56 ng/ml of DHP is sufficiently low for carry out pharmacokinetic studies to obtain realistic PK parameter. The extraction method gave consistent and reproducible recoveries for analytes from rabbit plasma, with no interference and matrix suppression. The results of validation indicate that method can be considered suitable for carrying out preclinical pharmacokinetic studies of DHP in rabbits

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