Determination of isotretinoin in human plasma: Application to pharmacokinetic study

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Abstract A simple, rapid, sensitive and accurate HPLC–UV detection method was developed and validated for the determination of isotretinoin in human plasma. The method involves protein precipitation of plasma by acetonitrile using nifedipine as the internal standard (IS). Chromatographic separation was achieved on Agilent C18 column using an acetonitrile: aqueous 0.5% glacial acetic acid (70:30, v/v) mobile phase. Isotretinoin in the eluent was monitored at 350 nm. Inter-assay of the coefficient of variations (CV%) over the range of plasma calibration curves (0.02–0.60 g/ml) ranged from 2.32% to 6.49% with good correlation coefficient ($R^2 = 0.999$). The average recovery of the extraction procedure was 99.128%. The LLOD and LLOQ are 0.007 and 0.02 g/ml, respectively. This method was applied for pharmacokinetic study and the results indicated that this method is suitable for pharmacokinetic and bioavailability studies.

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1. Introduction

Isotretinoin (INN) (13 cis)-retinoic acid, Fig. 1. Its medication is used for the treatment of severe acne and a number of cancers and rosacea. It was first developed to be used as a chemotherapy medication for the treatment of brain cancer and pancreatic cancer. Isotretinoin contains a carboxylic acid moiety and is a potentially teratogenic compound with reported terminal elimination half-lives in the order of 6–22. There is also interest in the prophylactic and therapeutic effects of 13-cis-RA in in vitro and in vivo carcinogenetic studies, pigmentary disorders and photoaging.

Several chromatographic methods have been published for the quantification of isotretinoin in a biological matrix (plasma or serum) and brain tissue. Some of these methods include high-performance liquid chromatography with ultra-violet detection and others are high-performance liquid chromatography with mass spectrometric detection. Most of these methods depend on lengthy sample preparation schemes including extractions with an organic solvent, evaporation of solvent and reconstitution prior to HPLC analysis or require expensive instruments such as LC–MS–MS or need column switching techniques (online solid-phase extraction). The purpose of this study was to establish a simple, rapid, sensitive and validated method for the determination of isotretinoin using...
nifedipine as the internal standard in human plasma and its application in pharmacokinetic study.

2. Experimental procedures

2.1. Chemicals and reagents

Isotretinoin working standard (99.9%) kindly provided by Medizen Co. (Egypt). Nifedipine (IS, 99.8%) was obtained from Sigma–Aldrich Co (UK). HPLC grade acetonitrile was purchased from Merck (Darmstadt, Germany). Human plasma was supplied by VACSERA, Egypt. Glacial acetic acid was purchased from Loba Chemie (India). Water was obtained from a Milli-Q water purification system (Millipore Co, France). The mobile phase component was filtered through a 0.45 μm Whatman membrane filter prior to its use.

2.2. Instruments and chromatographic conditions

Liquid chromatographic separation with UV detection was performed on a Waters chromatographic system (Milford, USA) equipped with pump controlled by Waters 610 controller, Waters 717 autosampler injector, and Waters 486 variable wavelength UV detector. The drug analysis data were acquired and processed using Empower software operated by Pentium III (450 MHz) processor (Lenovo, UK). The mobile phase consisted of a mixture of acetonitrile and 0.5% glacial acetic acid (70:30, v/v) pumped at flow rate of 1.5 ml/min. through the Agilent Eclipse XDB C_{18} 5 μm (150 × 4.0 mm, id) column at ambient temperature with a guard column C_{18} 5 μm 100 Å. Peaks were monitored by a UV detector adjusted at 350 nm. Quantification of isotretinoin was obtained by plotting isotretinoin to internal standard peak area ratios as a function of concentrations.

2.3. Calibration standards

Stock solutions of each of the isotretinoin and nifedipine (I.S) samples were prepared in acetonitrile at a concentration of 100.00 µg/ml and stored at 4 °C. Handling and analysis of all samples were performed under diffused light conditions (prepared away from light by using opaque glasses and aluminum foil). Evaluations of the assay were performed by six point calibration curves made by serial dilution of the stock solution of the drug at the nominal concentration range of 0.02–0.60 µg/ml of isotretinoin in human plasma. Slope and intercept of the calibration lines were determined.

2.4. Sample preparation

To each 400 µl of human plasma spiked with drug and I.S (50 µl from 100.00 µg/ml), 1.5 ml of acetonitrile was added for deproteinization. The tube was capped and the contents were vortexed for 30 s, and centrifuged for 10 min at 3500 rpm. The supernatant (200 µl) was injected into the HPLC system.

2.5. Method validation

Evaluation of the reversed-phase liquid chromatography methods was based mainly on proportionality (linearity assay), precision and accuracy.22 This method was developed and validated as per FDA guidelines.23

2.5.1. Selectivity

The interference by the endogenous compound was assessed by comparing the chromatograms obtained from the samples containing isotretinoin and the internal standard with those obtained from the blank samples.

2.5.2. Accuracy, precision and recovery

The intra-day precision of the assay was assessed by calculating the coefficients of variation (CV%) of the samples in six replicates and the inter-day precision was determined through the analysis of the samples on three consecutive days. Accuracy was determined by comparing the calculated concentrations to known concentrations with calibration curves.

The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard.

2.5.3. Calibration/standard curve

2.5.3.1. Linearity. The linearity of the calibration curve for isotretinoin was assessed in the range of 0.02–0.60 µg/ml in the plasma samples. The straight line regression was presented with its correlation coefficient.

2.5.3.2. Lower Limit of quantification (LLOQ). It is defined as the lowest concentration of the analyte that can be determined with acceptable precision and accuracy under the stated experimental conditions.

It is determined as the concentrations with a signal-to-noise ratio of 10:1, by comparing test results from samples with known concentrations of the analyte to blank samples. Each concentration standard should meet the following acceptable criteria: a precision of 20% and accuracy of 80–120%. The LLOQ was 0.02 µg/ml for isotretinoin in 18 standard calibration curves.

2.5.4. Stability

Low, medium and high quality control samples (n = 6), corresponding to 0.05, 0.20 and 0.60 µg/ml were retrieved from the deep freezer after three freeze–thaw cycles according to the clinical protocols. Samples were frozen at −80 °C in three cycles of 24, 48, and 36 h. In addition, the long-term stability of isotretinoin in quality control samples was also evaluated by analysis after 55 days of storage at −80 °C. Autosampler stability was studied following a 24 h-storage period in the autosampler tray. Bench top stability was studied for a 6 h period. Stability samples were processed and extracted along with the freshly spiked calibration curve standards. The precision and
accuracy for the stability samples must be within \( \pm 15\% \) and \( \pm 15\% \), respectively, of their nominal concentrations.

### 2.6. Clinical protocol

This method was applied in the analysis of plasma samples after the administration of a single dose of two capsules of isotretinoin 20 mg (Isotretinoin capsule) to healthy male volunteers. The study protocol was approved by the Ethics Committee of Bioavailability Studies (NODCAR). The age of 6 volunteers ranged from 29 to 38 years, and the subjects had a body weight ranging from 58 to 71 kg. All subjects gave their written informed consent. The study was conducted in accordance with the provisions of the Declaration of Helsinki. After an overnight fast period volunteers received a single dose of two capsules of isotretinoin 20 mg (Isotretinoin capsule) with 200 ml of water. Blood samples (3 ml) from a suitable antecubital vein were collected into heparin-containing tubes immediately before dose (0.0) and at 1, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 6.0, 8.0, 10.0 and 12.0 h post dosing. The blood samples were centrifuged at 3000 rpm for 5 min at room temperature and the plasma was removed and stored at \(-80\,^\circ\text{C}\) until assayed for its isotretinoin content. All samples from a single vol-

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**Table 1** Precision and accuracy of standard calibration curves of isotretinoin (intra-day assay) in human plasma \((n = 6)\).

<table>
<thead>
<tr>
<th>Theoretical concentration ((\mu g/ml))</th>
<th>Mean concentration found ((\mu g/ml))</th>
<th>S.D.</th>
<th>CV%</th>
<th>Accuracy%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.020</td>
<td>0.019</td>
<td>0.0004</td>
<td>2.11</td>
<td>95.00</td>
</tr>
<tr>
<td>0.050</td>
<td>0.046</td>
<td>0.0020</td>
<td>4.35</td>
<td>92.00</td>
</tr>
<tr>
<td>0.100</td>
<td>0.093</td>
<td>0.0030</td>
<td>3.23</td>
<td>93.00</td>
</tr>
<tr>
<td>0.200</td>
<td>0.201</td>
<td>0.0020</td>
<td>1.00</td>
<td>100.50</td>
</tr>
<tr>
<td>0.400</td>
<td>0.396</td>
<td>0.0050</td>
<td>1.26</td>
<td>99.00</td>
</tr>
<tr>
<td>0.600</td>
<td>0.606</td>
<td>0.0060</td>
<td>0.99</td>
<td>101.00</td>
</tr>
</tbody>
</table>

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**Figure 2** Chromatograms of (A) blank human plasma; (B) blank human serum spiked with isotretinoin (0.60 \(\mu g/ml\)) and nifedipine (12.50 \(\mu g/ml\)) and (C) plasma sample obtained from volunteers 3 h after oral administration of Isotretinoin capsules (2 capsules of 20 mg/capsule).
untente were analyzed in the same run in order to avoid inter-assay variations.

2.7. Pharmacokinetic analysis

Pharmacokinetic parameters from the human plasma samples were calculated by a noncompartmental statistics model using MINITAB® Software Release 13.1.

Blood samples were taken for a period of 3 to 5 times the terminal elimination half-life ($t_{1/2}$) and it was considered as the area under the concentration–time curve (AUC) ratio higher than 80% as per FDA guidelines.24,25

The first-order terminal elimination rate constant ($K_\text{el}$) was estimated by linear regression from the points describing the elimination phase on a log-linear plot. The maximum observed plasma concentration ($C_{\text{max}}$) and the time taken to achieve this maximum level ($T_{\text{max}}$) were obtained directly from the curves. The areas under the curve for isotretinoin plasma concentration versus time for 0–12 h (AUC$_{0-12}$ h) were calculated by applying the linear trapezoidal method. The extrapolation of this area to infinity (AUC$_{0-\infty}$) was obtained by adding the value $C_{12}/K_\text{el}$ to the calculated AUC$_{0-12}$ h where $C_{12}$ is the isotretinoin plasma concentration at 12 h and $K_\text{el}$ is the first-order terminal elimination rate constant.

3. Results and discussion

Owing to the photolability and sensitivity to heat and oxidation of the retinoids, extreme precautions to protect isotretinoin from white light and to minimize exposure to oxygen need to be taken when handling isotretinoin and the respective solution.

The present study describes a simple, sensitive, accurate and reproducible HPLC method for the determination of isotretinoin in human plasma. This method has several advantages over the previously reported methods. Sample preparation, in this method, is simpler as it involves only one step of deproteinization and requires a relatively small volume of plasma (0.4 ml). In contrast, the previous methods are characterized by lengthy sample preparation schemes including extractions with an organic solvent, evaporation of solvent and reconstitution prior to HPLC analysis.9–11,16,17,20,21 Lyophilization of the sample before extraction is required in other methods.3 The use of HPLC–UV with column switching technique (online solid-phase extraction) appeared to be the most promising.12,14,15 However, a complicated processing technique may limit its wide application. Moreover the very low quantification limit obtained with a UV detector allowed us to avoid using fluorimetric or mass spectrophotometric detection19–21, which requires more expensive equipment and makes our method particularly useful for pharmacokinetic studies.

Different ratios of the mobile phase were tried and this ratio was the suitable one. System suitability testing of the HPLC method gave good resolution ($R$) = 10.867; relative retention time ($a$) = 24.83; column capacity ($K$) = 10.4 and tailing factor ($T$) = 1.2. Under the chromatographic conditions described, isotretinoin and nifedipine (IS) peaks are well resolved. Fig. 2 shows typical chromatograms of blank plasma in comparison to plasma spiked with drug and the internal standard and the plasma sample obtained at 3 h from volunteers who received a single oral dose. The retention times of the internal standard and isotretinoin were 1.3 min and 10.8 min, respectively. No endogenous compounds appear at the retention time of isotretinoin or of the IS to interfere with their peaks. The base line was relatively free from drift. Six concentrations defined the calibration curves. A calibration curve was obtained by plotting the peak-area ratio (drug/IS) against the concentration of isotretinoin in plasma. The linearity of the calibration curves ($n = 18$) was verified from 0.02 to 0.60 µg/ml and the corresponding regression equation was $y = 1.051x - 0.008$, $r^2 = 0.999$, where $y$ is the peak area ratio of isotretinoin to nifedipine, $x$ is the concentration of isotretinoin (µg/ml) in plasma and $r$ is the correlation coefficient.

Intra-day precision (CV%) and accuracy were determined with six calibration curves of six points of concentration (analysis in the same day) with the range of 0.99–4.35% and 92.00–101.00, respectively (Table 1). Inter-day assay precision and the accuracy of standard curves were determined by comparing the peak area ratio of 6 standard curves prepared and injected on 3 different days with the ranges of 1.98–8.08% and 95.00–101.00, respectively (Table 2).

The extraction recovery determined for isotretinoin was shown to be consistent, precise and reproducible. The average recovery was 99.128% which is acceptable for the routine measurement of isotretinoin.

Table 3 summarizes the freeze and thaw stability, short-term stability, long-term stability and auto sampler stability.

| Table 2 | Precision and accuracy of standard calibration curves of isotretinoin (inter-day) in human plasma ($n = 18$). |
|---|---|---|---|---|
| Theoretical concentration ($\mu$g/ml) | Mean concentration found ($\mu$g/ml) | S.D. | CV% | Accuracy% |
| 0.020 | 0.019 | 0.0008 | 4.21 | 95.00 |
| 0.050 | 0.048 | 0.0020 | 4.17 | 96.00 |
| 0.100 | 0.099 | 0.0080 | 8.08 | 99.00 |
| 0.200 | 0.202 | 0.0040 | 1.98 | 101.00 |
| 0.400 | 0.398 | 0.0100 | 2.51 | 99.50 |
| 0.600 | 0.600 | 0.0180 | 3.00 | 100.00 |

| Table 3 | Data showing the stability of isotretinoin in human plasma at different QC levels ($n = 6$). |
|---|---|---|---|
| Accuracy (mean ± CV%) | 0.05 µg/ml | 0.20 µg/ml | 0.60 µg/ml |
| Short-term stability (6 h, room temperature) | 108.27 ± 7.40 | 89.42 ± 5.04 | 88.57 ± 8.04 |
| Freeze and thaw stability (3 cycles, −80 °C-room temperature) | 107.72 ± 4.56 | 89.41 ± 6.10 | 90.85 ± 6.93 |
| Long-term stability (55 days, −80 °C) | 105.35 ± 5.92 | 101.48 ± 3.62 | 99.47 ± 3.61 |
| Auto sampler stability (24 h, 4 °C) | 101.10 ± 4.52 | 99.87 ± 6.52 | 98.38 ± 4.25 |
data of isotretinoin. All the results showed the reliable stability behavior during these tests and there were no stability-related problems during the routine analysis of samples for the bioavailability study.

The method described here was successfully applied to pharmacokinetic study of isotretinoin in healthy human volunteers. General adverse reactions were not observed with any volunteer. Following oral administrations, plasma concentration–time curves of isotretinoin best fit a non-compartment model in all subjects. Plasma concentration–time curves are shown in Fig. 3. The pharmacokinetic parameters estimated are shown in Table 4.

4. Conclusion

A simple HPLC–UV method with good accuracy, precision and low detection limit was developed for the determination of isotretinoin in human plasma samples. Lyophilization of the sample before extraction or the use of expensive equipments as LC/MS is not necessary in the present work and thus the method is much faster and economical. Ultimately, the validated HPLC method employed here exhibited acceptable precision and adequate sensitivity for the quantification of isotretinoin in human plasma samples resulting from the clinical pharmacokinetic and bioavailability studies. The median $T_{\text{max}}$, $C_{\text{max}}$ and AUC values were similar to those reported in the literature.

Table 4 Pharmacokinetic parameters required for assessment of Isotretinoin bioavailability.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ (µg h/ml)</td>
<td>0.434 ± 0.033</td>
</tr>
<tr>
<td>$t_{\text{max}}$ (h)</td>
<td>1.75</td>
</tr>
<tr>
<td>AUC_{0-12} (µg h/ml)</td>
<td>2.480 ± 0.014</td>
</tr>
<tr>
<td>AUC_{0-inf} (µg h/ml)</td>
<td>3.097 ± 0.393</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>4.604 ± 1.57</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>7.703 ± 2.269</td>
</tr>
<tr>
<td>$K_{\text{cl}}$ (h)</td>
<td>0.1599 ± 0.054</td>
</tr>
</tbody>
</table>

Figure 3 Mean drug plasma concentration–time curve of isotretinoin from 6 volunteers after oral administration of Isotretinoin capsule (2 capsules of 20 mg/capsule).

References


