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# Determination of calcium dobesilate in human plasma using ion-pairing extraction and high-performance liquid chromatography

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**Abstract**

A rapid, simple reversed-phase high-performance liquid chromatographic method with ultraviolet absorbance detection has been developed for the determination of calcium dobesilate in human plasma. Sample processing is based on an ion-pairing extraction with tetra-*n*-butylammonium hydroxide as cationic pairing ion and dichloromethane. Separation of the investigated calcium dobesilate and 2,4-dihydroxybenzoic acid as internal standard was achieved on a Discovery RP-Amide $C_{16}$ analytical column with 50 m*M*, pH 2.5, potassium dihydrogenphosphate buffer–acetonitrile (75:25, v/v) mobile phase. The wavelength was set at 305 nm. The limit of quantitation is 100 ng/ml and the calibration curve is linear up to 50 μg/ml. Within-day and between-day precision expressed as the relative standard deviation is about 10% and the accuracy of the determination did not deviate from 100% by more than ±10%. The developed method was found to be suitable for application in human bioequivalence studies. 

*Keywords:* Calcium dobesilate

## 1. Introduction

Calcium dobesilate (Fig. 1) is a vascular protectant frequently used in general medicine and ophthalmology. It is mainly used to treat diabetic retinopathies and other vasculopathies. In spite of the fact that for more than 25 years calcium dobesilate has been accepted in the treatment of certain venous insufficiencies, particularly diabetic retinopathy, until now there have only been a few studies on the HPLC methods for measuring calcium dobesilate in blood. Benakis et al. [1] measured the calcium dobesilate

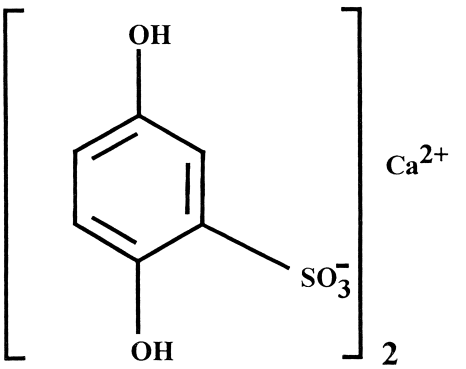


Fig. 1. Structural formula of calcium dobesilate (DBZ).

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$^{35}S$ blood level after a 500 mg oral dose in humans using a liquid scintillation technique. Franke et al. [2] used only a protein precipitation step (with acetonitrile) as sample preparation after the supernatant was injected into the chromatograpic system. They used a reversed-phase HPLC method [2] for the bioavailability studies of calcium dobesilate in humans after a 500 mg dose. We tried this method, but according to our results the limit of quantitation was found to be 2 μg/ml plasma. In addition, we had doubts about the lifetime of the analytical column. (In a bioequivalence study there are about 1000 samples.) We found reference to one more HPLC method in the literature [3,4] for the determination of calcium dobesilate in dog plasma, but in the papers of Plessas et al. [3,4] we did not find any information about sample preparation. Under the heading 'Determination of calcium dobesilate' they only described the HPLC conditions. They did not use an internal standard.

The aim of this work was to develop and validate a specific high-performance liquid chromatographic (HPLC) method for the determination of calcium dobesilate (Fig. 1) in human plasma for use in a bioequivalence study. This assay was designed in order to quantify calcium dobesilate in human plasma following a 500 mg oral dose of the drug. The HPLC method developed in this study was employed in a simple liquid–liquid extraction using an ion-pairing counter-ion in the sample processing. In the separation we used a Discovery column which provided exceptional reproducibility without a modifier in the mobile phase. During the determinations, 2,4-dihydroxybenzoic acid served as internal standard.

## 2. Experimental

### 2.1. Chemicals

Calcium dobesilate (DBZ) (2,5-dihydroxybenzenesulfonic acid calcium salt) was provided by Claus Huth Praha (Praha, Czech Republic). 2,4-Dihydroxybenzoic acid was used as internal standard (I.S.) (Aldrich, St. Louis, MO, USA). Methanol, tetrabutylammoniumhydroxide (20%) and 85% phosphoric acid (gradient grade) were from Merck (LiChrosolv grade) (Darmstadt, Germany). Dichloromethane was purchased from Sigma (Riedel-de Haen) (St. Louis, MO, USA). Acetonitrile was from Scharlau (supragradient HPLC grade) (Spain). Sodium hydroxide and potassium dihydrogenphosphate were products of Reanal Fine Chemicals (Budapest, Hungary). The chemicals used were of analytical grade.

### 2.2. Chromatographic conditions

A Shimadzu 10A chromatographic system (LC-10 AD pump, SIL-10A autosampler, SCL-10A system controller, SPD-10A UV detector, CTO-10A column thermostat, all made by Shimadzu, Kyoto, Japan) was used for liquid chromatography with Class VP (Ver. 4.3) chromatographic software.

Separation was performed at 23°C (column thermostat) using a Supelcosil Discovery RP-Amide $C_{16}$ 5 μm (150×4.6 mm) analytical column equipped with a Discovery (20×4.6 mm) guard column. Both were obtained from Supelco (Bellefonte, PA, USA). The flow-rate was 1 ml/min.

For UV detection the wavelength was 305 nm. The eluent, 50 m*M* potassium dihydrogenphosphate buffer (pH 2.5)–acetonitrile (75:25, v/v), was filtered through Supelco Nylon (0.45 μm) and degassed by ultrasonication.

### 2.3. Preparation of standard solutions

Stock solutions of 1 mg/ml concentration were prepared from DBZ and from I.S. with doubly distilled water. Both stock solutions were stable for at least 4 weeks at −20°C. Dilutions of the stock solutions to obtain 10 and 1 μg/ml DBZ working standard solutions were made with doubly distilled water freshly prepared each week and stored at 4°C. Human plasma was obtained from drug-free blood containing 1 mg EDTA-sodium salt per milliliter whole blood. The anticoagulant-containing blood was centrifuged at 1500 rpm for 10 min and the resulting plasma was stored at −20°C until use. The biological samples were prepared from drug-free

plasma with the addition of working standard solution.

### 2.4. Sample processing

For the determination of DBZ plasma concentrations, deep-frozen plasma samples were allowed to thaw at room temperature. I.S. (50 μg) was then added to 1 ml of plasma, using the 1 mg/ml concentration stock solution, and then homogenized with a vortex mixer for 5 s. One milliliter of [80% (v/v) 0.2 *M* potassium dihydrogenphosphate (pH 2.5) and 20% (v/v) tetrabutylammoniumhydroxide mixture] ion-pairing buffer was added to the plasma and vortexed for 5 s. DBZ was then extracted from the plasma using liquid–liquid extraction with 8 ml dichloromethane. The resultant mixture was mixed and extracted for 10 min at 250 rpm using a Heidolph shaker and centrifuged at 1500 rpm (ca. 1000 *g*) for 10 min at 4°C. Six milliliters of the upper organic layer was transferred to a clean dry glass tube and was evaporated to dryness at 37°C under a stream of nitrogen. To the resulting residue was added 200 μl of the mobile phase, and the solution was vortex-mixed for 45 s. Aliquots of 50 μl were injected into the chromatographic system.

#### 2.4.1. Calibration curves

Calibrations were performed with freshly spiked samples as follows: 1 ml was spiked with an appropriate volume of DBZ working standard solution to obtain plasma concentrations of 0.1, 0.5, 1, 2, 5, 10, 20, 25, and 50 μg/ml. To each sample, 50 μg I.S. was added. Extraction and liquid chromatography were carried out according to the procedure described above. At each concentration, two parallel determinations were carried out per validation day.

Calibration curves were fitted to the measured peak area ratios by the least squares method applying the Class VP 4.3 sw. program, which generated an equation for the calibration curve. Variances of the peak area ratios were tested to determine whether or not it was necessary to perform a weighted regression analysis. The DBZ concentration of study samples was calculated using the $1/x^2$ weighting method from the area ratio of the chromatographic peaks of DBZ and I.S. using the equation of the daily calibration curve and expressed as μg/ml of plasma.

#### 2.4.2. Quality control samples

For method validation, QC samples were prepared in advance at 2, 10, and 20 μg DBZ/ml plasma concentrations and were stored in 1 ml aliquots at −20°C. The I.S. was added to each QC sample just before sample processing (50 μg/ml).

## 3. Results and discussion

### 3.1. Separation

With the present method, the mobile phase [50 m*M* potassium dihydrogenphosphate buffer (pH 2.5)–acetonitrile (75:25, v/v)], together with the Discovery RP-Amide $C_{16}$ stationary phase, ensured a symmetrical, close to optimum peak shape for both the study compound and the I.S. Drug-free plasma samples from six volunteers were analysed to determine significant interfering endogenous plasma components. Fig. 2 shows the chromatogram of a typical blank plasma extract obtained using liquid–liquid extraction. Endogenous compounds interfering with the retention of DBZ or that of the I.S. cannot be seen in the chromatograms; this is evidenced by the lack of interfering endogenous peaks in the plasma samples for DBZ and I.S. Fig. 3 shows the chromatogram of a plasma sample 3 h after administration of 500 mg DBZ to a volunteer.

### 3.2. Method validation

Method validation was carried out according to internationally accepted criteria [5–7]. Tables 1–4 contain detailed results of method validation.

#### 3.2.1. System suitability

For the examination of system suitability, five injections on the same day were made from the same biological sample containing 20 μg/ml DBZ and 50 μg/ml I.S. According to the results of system suitability tests, the average retention times of DBZ and the internal standard were 4.34 and 8.57 min, respectively. On the basis of six parallel determi-

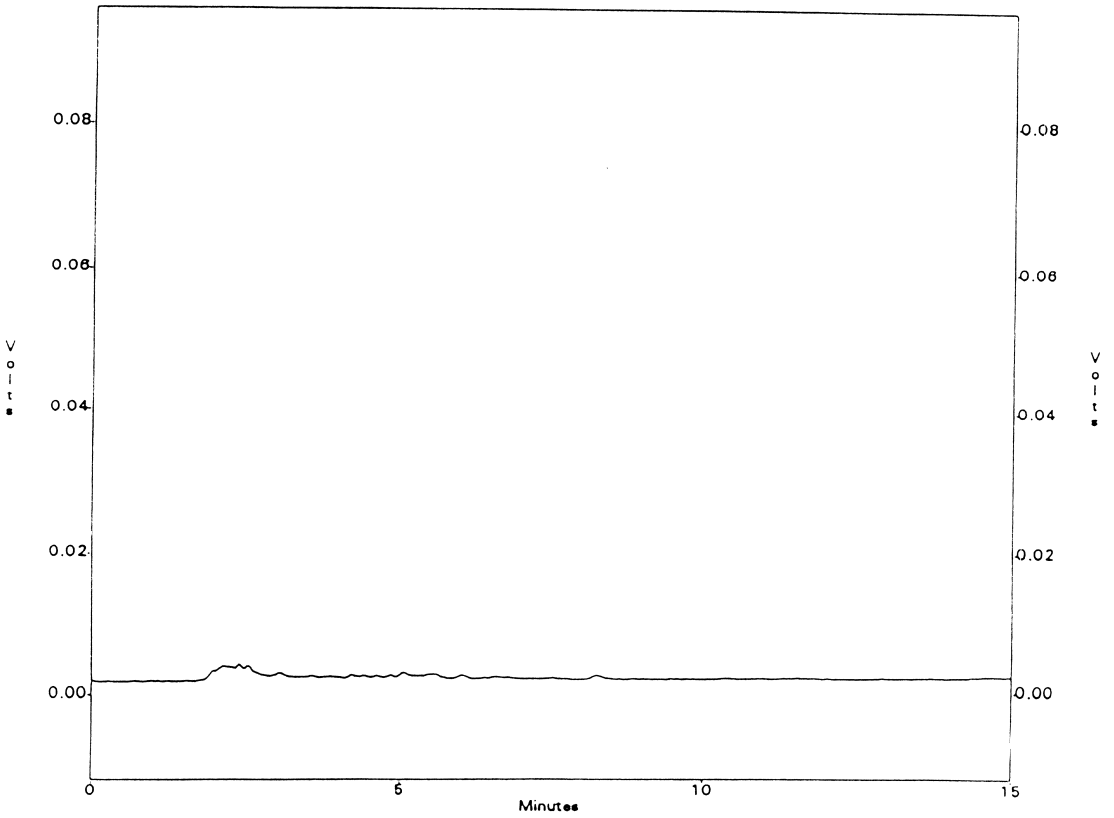


Fig. 2. Chromatogram of a characteristic blank plasma extract. Chromatographic conditions are described in the text.

nations, the precision of injection calculated from peak area values was 0.51% for DBZ and 2.33% for the I.S. For the retention time, the values are 0.78% (DBZ) and 0.51% (I.S.), respectively.

*3.2.2. Limit of quantitation*

The limit of quantitation (LOQ) was defined as the lowest concentration with a relative standard deviation (RSD) of less than 20%. Validation data indicated that the LOQ was 0.10 μg/ml and the limit of detection (LOD) was 0.03 μg/ml.

*3.2.3. Linearity of calibration*

Calibration curves with good linearity were obtained from the calibration points using least squares linear regression (weighted calibration curves, weighting factor, $w = 1/x^2$) in the range 0.1–50 μg/ml. The correlation coefficient ($r$) values were in the range 0.9991–0.9999.

*3.2.4. Intra- and inter-day precision and accuracy*

The intra- and inter-day precision and accuracy of the method were determined using quality control (QC) samples at three different concentrations (2, 10, and 20 μg/ml). Five parallel determinations were made at each concentration (Table 1).

The inter-day precision and accuracy of the method was calculated by the analysis of QC samples on 3 different days. Fifteen parallel determinations were made at each concentration (Table 2).

As indicated by the results of intra-day and inter-day precision and accuracy determinations with QC samples, the RSD values remained below ±15% and the accuracy of the determination did not deviate from 100% by more than ±15% (Tables 1 and 2).

*3.2.5. Stability study*

The stability of DBZ in plasma samples at three different concentrations (2, 10, and 20 μg DBZ/ml

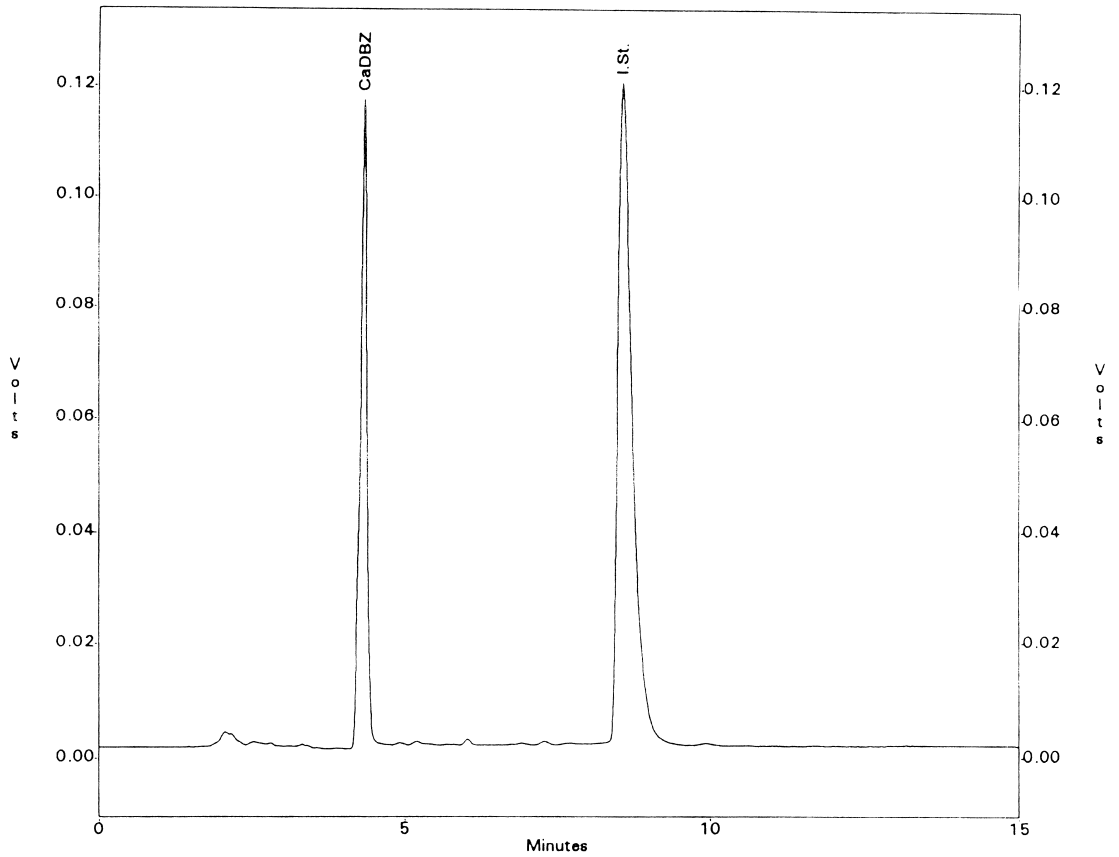


Fig. 3. Chromatogram of a typical extract of a plasma sample from a volunteer 3 h after administration of 500 mg calcium dobesilate. The measured concentration was 9.10 μg/ml. Chromatographic conditions are described in the text.

plasma) and stored at −20 and −70°C was examined after 2 and 5 weeks of storage. At all three concentrations and for each storage period (0, 2 and 5 weeks) two parallel determinations were made.

DBZ did not show any significant decomposition in human plasma during 2 weeks of storage at −20°C but, unfortunately, it did between 2 and 5 weeks of storage. Three sets of samples were stored in a freezer at −70°C for 5 weeks. The concentrations found were within the allowed limit of the

Table 1
Intra-day precision and accuracy data for calcium dobesilate in human plasma

| Nominal conc. (μg/ml) | 1st day ($n=5$) | | | | 2nd day ($n=5$) | | | | 3rd day ($n=5$) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean calc. conc. (μg/ml) | ±SD | RSD (%) | Accuracy (%) | Mean calc. conc. (μg/ml) | ±SD | RSD (%) | Accuracy (%) | Mean calc. conc. (μg/ml) | ±SD | RSD (%) | Accuracy (%) |
| 2 | 1.95 | 0.052 | 2.7 | 97.5 | 1.96 | 0.034 | 1.7 | 98.0 | 2.12 | 0.036 | 1.7 | 106.0 |
| 10 | 9.80 | 0.071 | 0.7 | 98.0 | 9.84 | 0.17 | 1.7 | 98.4 | 10.07 | 0.0630 | 0.6 | 100.7 |
| 20 | 19.48 | 0.430 | 2.2 | 97.4 | 19.10 | 0.216 | 1.1 | 95.5 | 19.64 | 0.523 | 2.7 | 98.2 |

Table 2
Inter-day precision and accuracy data for calcium dobesilate in human plasma

| Validation days 1–3 | | | |
|---|---|---|---|
| Theoretical conc. (μg/ml) | 2 | 10 | 20 |
| Mean calc. values (μg/ml) | 2.07 | 9.90 | 19.41 |
| ±SD | 0.089 | 0.16 | 0.450 |
| RSD (%) | 4.3 | 1.6 | 2.3 |
| Accuracy (%) | 103.5 | 99.0 | 97.1 |
| *n* | 15 | 15 | 15 |

nominal concentration, indicating no significant loss during storage at −70°C. The results are presented in Table 3.

The autosampler stability of three sets of QC samples was determined over 24 h. The results of this part of the stability study indicate that the QC samples remained stable in a dark autosampler for 24 h. Stability tests demonstrated that DBZ and the I.S. remained stable for at least 5 weeks in stock solution.

### *3.2.6. Determination of recovery*

One milliliter plasma samples containing 2, 10, and 20 μg of DBZ but no I.S. were processed by liquid–liquid extraction. Internal standard (50 μg) was added to the sample after extraction when the dry residue was dissolved with mobile phase. Three parallel determinations were carried out at each concentration. The peak area ratios obtained with the extracted samples were compared with those of an aqueous solution containing the same concentration of DBZ and the I.S. The concentration dependence of recovery was negligible (Table 4); the average extraction efficiency was 84.0%.

Table 3
Result of stability tests for calcium dobesilate

| Temp. (°C) | Nominal conc. (μg/ml) | 2nd week | | 4th week | |
|---|---|---|---|---|---|
| | | Mean calc. conc. (μg/ml) | Accuracy (%) | Mean calc. conc. (μg/ml) | Accuracy (%) |
| −20 | 2 | 2.13 | 106.5 | 1.81 | 90.5 |
| | 10 | 9.76 | 97.6 | 9.43 | 94.3 |
| | 20 | 19.02 | 95.1 | 17.70 | 88.5 |
| Mean accuracy | | | 99.7 | | 91.1 |
| | | 2nd week | | 5th week | |
| −70 | 2 | 1.93 | 96.5 | 1.89 | 94.5 |
| | 10 | 10.26 | 102.6 | 10.35 | 103.5 |
| | 20 | 19.81 | 99.1 | 20.20 | 101.0 |
| Mean accuracy | | | 99.4 | | 99.7 |

Table 4
Recovery of calcium dobesilate from plasma

| Nominal conc. (μg/ml) | DBZ/I.S. peak area ratio in plasma extract[a] (*n* = 3) | DBZ/I.S. peak area ratio in aqueous solution of the same concentration (*n* = 3) | Recovery (%) |
|---|---|---|---|
| 2.0 | 0.0268 | 0.0330 | 81.2 |
| 10.0 | 0.0550 | 0.0629 | 87.4 |
| 20.0 | 0.1229 | 0.1473 | 83.4 |
| Mean | | | 84.0±3.1 |

[a] I.S. was added to the sample after extraction.

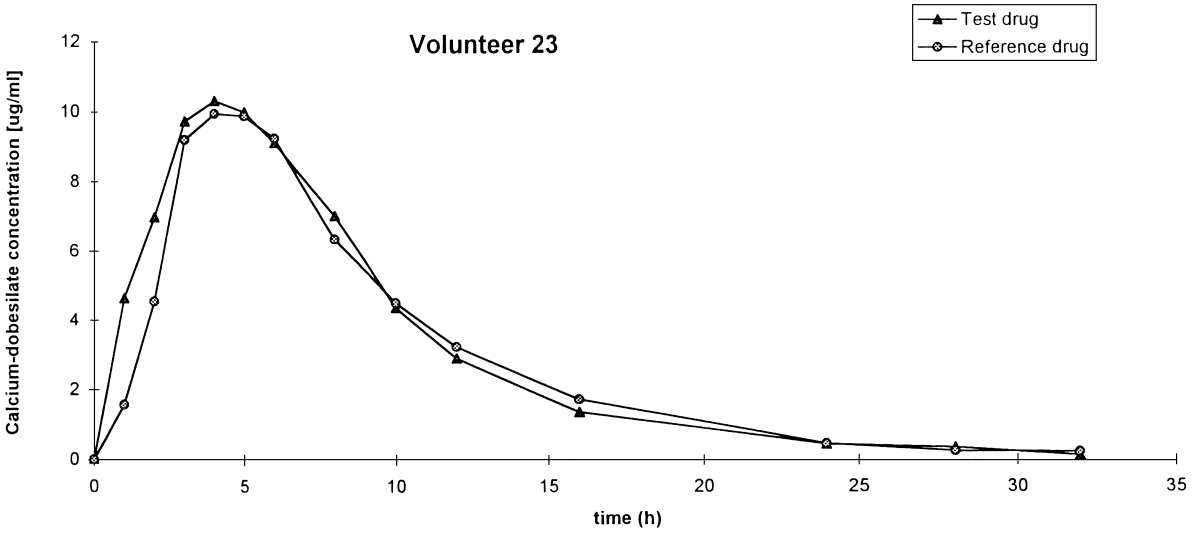


Fig. 4. Concentration–time curves for DBZ obtained from a volunteer after the oral administration of two different preparations containing 500 mg calcium dobesilate.

### *3.3. Application to a bioequivalence study*

The proposed method was found to be suitable for the determination of DBZ in human plasma for the purpose of a bioequivalence study. Fig. 4 shows the concentration–time curves for DBZ obtained from a volunteer after the oral administration of two different preparations containing 500 mg calcium dobesilate.

## 4. Conclusion

A liquid chromatographic method (HPLC) with UV detection has been elaborated and validated for the determination of calcium dobesilate in human plasma. Considering the 0.1 μg/ml plasma limit of quantitation, linear calibrations in the range 0.1–50 μg/ml and the precision (with the RSD within the 15% level acceptable for biological samples), the method was found to be suitable for the determination of calcium dobesilate in human plasma and for human pharmacokinetic (bioequivalence) studies.

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