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# Liquid chromatography/tandem mass spectrometry assay for the quantification of troxerutin in human plasma

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**A simple, rapid, sensitive and specific liquid chromatography/tandem mass spectrometry method was developed and validated to quantify troxerutin in human plasma. The analyte and rutin, used as the internal standard, were analyzed on a Phenomenex Synergi Fusion RP column interfaced with a triple-quadrupole tandem mass spectrometer using positive electrospray ionization. Acetonitrile/water (20:80 v/v) was used as the isocratic mobile phase, with 0.1% formic acid in water. A simple sample preparation method of protein precipitation with perchloric acid was employed. The assay was linear over the concentration range 31.25–4000 pg/mL. Correlation coefficients generated by linear regression with a $1/x^2$ weighting factor ranged from 0.9991 to 0.9996. The intra- and inter-day precision over the entire concentration range were less than 12.28%. The method was successfully applied to a pharmacokinetic study after oral administration of a 300 mg troxerutin drop pill to 18 healthy volunteers. Copyright © 2006 John Wiley & Sons, Ltd.**

Troxerutin, {2-[3,4-bis(2-hydroxyethoxy)phenyl]-3-[(6-deoxy-α-L-manno-pyranosyl)-β(-D-glucopyranosyl)-oxy]-5-hydroxy-7-(2-hydroxyethoxy)-4*H*-1-benzopyran-4-one}, a trihydroxyethyl derivative of the flavanoid rutin,[1,2] has been used therapeutically to treat chronic venous insufficiency,[3–10] varicosity, capillary fragility,[11–13] and abnormal leakage, and it has anti-erythrocytic, anti-thrombotic, fibrinolytic,[4] odema-protective,[14] and rheological activity.[5,8] Low plasma concentration is achieved following administration of troxerutin; thus a quantification method for the drug in pharmacokinetic studies and monitoring of its efficacy need to be sensitive and specific.

Few methods have been reported for determination of troxerutin in biological samples. Dittrich *et al*.[15] employed a high-performance liquid chromatography (HPLC) method to determine troxerutin in plasma and urine following oral administration in humans, but the lower limit of quantification (LLOQ) is too high.

In the present work, a simple and sensitive HPLC/tandem mass spectrometry (MS/MS) method for the quantification of troxerutin in human plasma, with rutin as the internal standard (IS), is described. Herein a simple sample preparation method of protein precipitation with perchloric acid was employed, which made it an attractive procedure in high-throughput bioanalysis. This method was applied to a pharmacokinetic study after an administration of 300 mg troxerutin to 18 healthy volunteers.

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## EXPERIMENTAL

### Chemicals

Troxerutin and rutin (IS) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile (HPLC-grade) was obtained from Merck (Darmstadt, Germany). Formic acid (HPLC-grade) was purchased from TEDIA Corporation (Fairfield, USA). Distilled water, prepared from demineralized water, was used throughout the study. Blank plasma was provided by The First Affiliated Hospital of Anhui Medical University (Hefei, China), and it was pooled from 20 fasted subjects.

### Instruments

A TSQ Quantum Ultra AM triple-quadrupole mass spectrometer (Thermo Finnigan), coupled with an electrospray ionization (ESI) source, a Finnigan Surveyor LC pump and Finnigan Surveyor autosampler, was used for LC/MS/MS analysis. Data acquisition was performed with Xcalibur 1.4 software (Finnigan).

### LC/MS/MS conditions

Chromatographic analysis was performed on a Phenomenex Synergi Fusion RP (100 × 2.0 mm i.d., 4 μm) column (Phenomenex, USA), which was protected by a Security Guard $C_{18}$, 5 μm (4 × 3.0 mm i.d.) guard column (Phenomenex, USA). The mobile phase composition was a mixture of water (containing 0.1% formic acid) and acetonitrile (80:20, v/v), which was pumped at a flow rate of 0.2 mL/min. A post-column divert valve was used to direct HPLC

eluate to a waste container in the first 2 min of the chromatographic run and afterwards to the ionization source. Mass spectrometric detection with the ESI source was performed in positive ion mode, using selected reaction monitoring (SRM). The precursor fragment ion reaction for troxerutin was *m/z* 743.2 → 435.0, and for rutin was *m/z* 610.7 → 303.0. The product ion mass spectra of $[M+H]^+$ ions of troxerutin and rutin are shown in Figs. 1(A) and 1(B). For both troxerutin and rutin, the following optimized parameters were obtained: capillary temperature of 340°C, electrospray voltage of 4800 V, nitrogen was used as sheath gas and auxiliary gas at the pressures (arbitrary units) of 27 and 3, respectively. At the same time, collision-induced dissociation (CID) was performed using argon at a collision gas pressure of 1.4 mTorr and collision energy was 22 eV in both cases. The scan width for SRM was *m/z* 0.1, and scan time was 0.5 s. The peak width settings for both Q1 and Q3 were 0.7u.

## Preparation of stock solutions and standards

A stock solution of troxerutin was prepared in acetonitrile at a concentration of 400 μg/mL, and a stock solution of the IS was prepared in acetonitrile at a concentration of 250 μg/mL. Calibration curves for troxerutin were prepared by spiking blank plasma at concentrations of 31.25, 62.5, 125, 250, 500, 1000, 2000 and 4000 pg/mL, and the analytes were prepared in triplicate for each concentration. All the solutions were

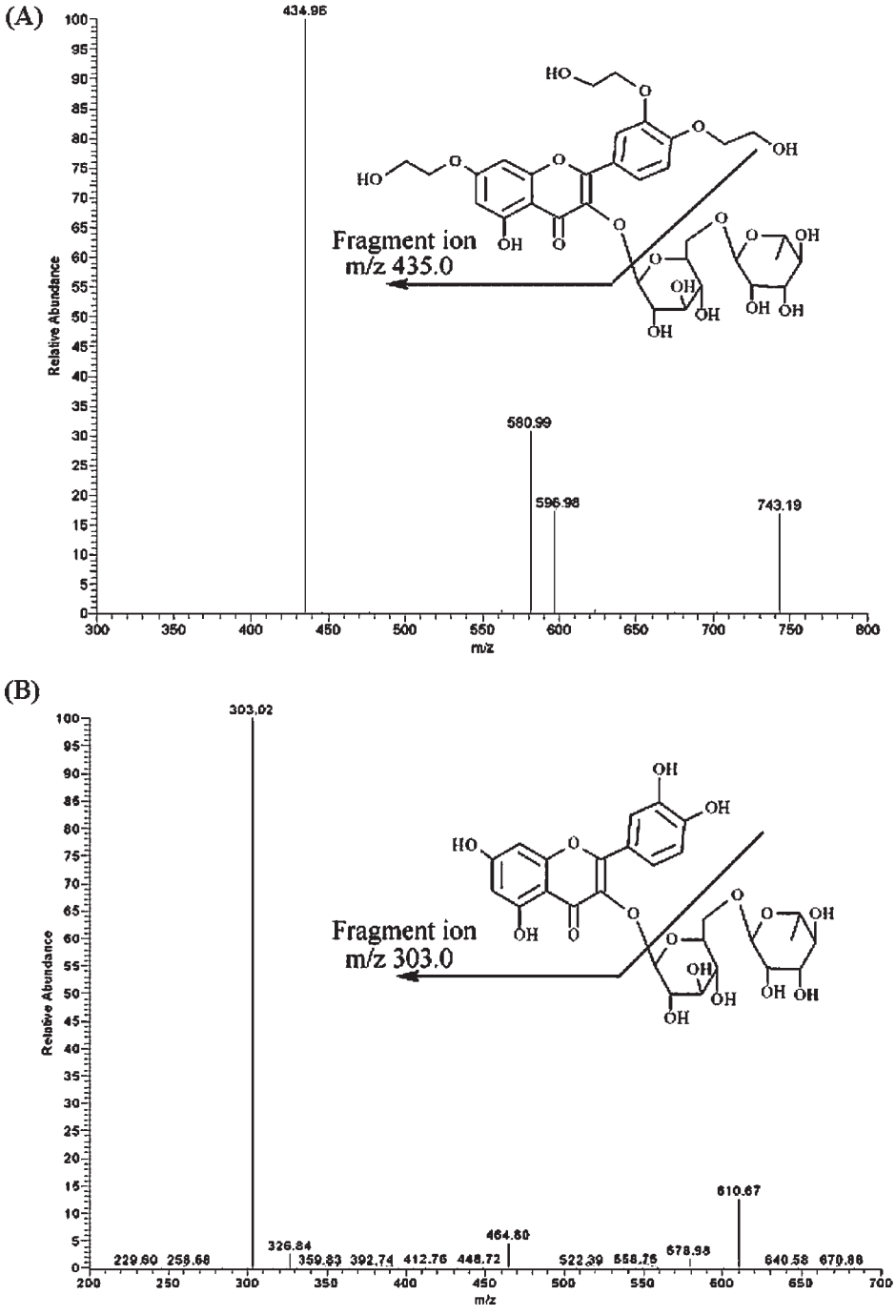


**Figure 1.** Product ion mass spectra of $[M+H]^+$ ions of troxerutin (A) and rutin (B).





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stored at −20°C and were brought to room temperature before use.

Quality control (QC) samples were prepared in quintuplicate at three different levels by spiking blank plasma at the concentrations of 62.5, 250 and 2000 pg/mL, representing low, medium and high concentration QC samples, respectively. The spiked plasma samples (standards and QC samples) were prepared freshly for each analytical batch both in the pre-study validation and along with the unknown samples.

### Extraction

QC samples, calibration standards and unknown plasma samples were all extracted as follows: 10 μL of IS solution (25 ng/mL rutin in acetonitrile) were spiked to a 100 μL aliquot of plasma sample. The sample mixture was briefly mixed. Then 80 μL perchloric acid (6%, v/v) were added to the mixture and vortex-mixed for 2 min and centrifuged for 10 min at 13400 *g* to remove precipitate. The supernatant was collected, and a 20 μL aliquot of solution was injected into the LC/MS/MS system for analysis.

### Assay validation

The method was validated for linearity, LLOQ, accuracy, precision, recovery and stability.

For the calibration standards, peak area ratios (troxerutin/rutin) were plotted against troxerutin plasma concentrations, and fitted by weighted ($1/x^2$) least-squares linear regression. To evaluate linearity, plasma calibration curves were prepared and assayed in triplicate on three separate days. In addition, a blank plasma was also analyzed to confirm absence of interferences.

Accuracy and precision were assessed in conjunction with the linearity studies by determining QC samples using quintuplicate ($n = 5$) preparations of spiked plasma samples at three concentration levels on three separate days. The accuracy, i.e., percentage concentration deviation, was expressed by (mean observed concentration – spiked concentration)/(spiked concentration) × 100%, and the precision was assessed in terms of the relative standard deviation (RSD) of the measured concentrations. The acceptable criterion was 15% or better.

The recoveries of troxerutin from the extraction procedure were evaluated by a comparison of the mean peak areas of QC samples at three levels to that of samples prepared by spiking extracted drug-free plasma samples with the same amounts of troxeruin at the step immediately prior to chromatography. These spike-after-extraction samples represented 100% recovery.

Owing to the components of the sample matrix, signal suppression or enhancement may occur. Matrix effect was calculated by comparing peak areas of troxerutin obtained from the spike-after-extraction samples with those from the unextracted pure standard solutions at the same concentrations.

Troxerutin stability in plasma was assessed by analyzing QC samples at concentrations of 62.5, 250 and 2000 pg/mL, respectively, in triplicate ($n = 3$), after exposure to different conditions of time and temperature. The results were compared with those for freshly prepared QC samples, and the percentage concentration deviation was calculated. For short-term stability, the plasma samples were kept at room temperature (20°C) for 4 and 12 h. The stability was also evaluated after storage of the plasma samples at –20°C for 7 days. The freeze/thaw stability was obtained after three freeze/thaw cycles on consecutive days.

### Pharmacokinetic study

The method was applied to analyze the plasma samples from 18 healthy male adult volunteers. The experimental protocol was approved by the institutional review board of the State Base for Drug Clinical Trial of Anhui Medical University (Heifei, China). Eighteen male subjects with an age range of 21–24 ($22.35 \pm 0.8$) years and weight range of 53–85 ($66.73 \pm 8.55$) kg enrolled in the study. Before enrollment and at the end of the study, each subject underwent a physical examination and clinical laboratory tests, including blood chemistry, hematology, and liver enzymes. After an overnight (more than 10 h) fast, subjects received a single oral dose of a 300 mg troxerutin drop pill (Beijing Chia Tai Green Continent Pharmaceutical Co., Ltd., China). Blood samples (3 mL) was taken by venepuncture into heparinized evacuated glass tubes prior to dosage and serially at 0.5, 1, 1.5, 2, 3, 4, 6, 10, 15, 24 and 36 h thereafter. Following standing for 30 min and centrifugation (4000 *g* for 10 min) the plasma was separated and stored at –20°C until analysis.

## RESULTS AND DISCUSSION

### LC/MS/MS conditions

Troxerutin has several kinds of hydroxyl group, thus producing good mass spectrometric responses in either positive or negative ionization mode. The signal intensities obtained in positive mode were much higher (about 10 times) than that in negative mode. The CID of the $[M+H]^+$ ion of troxerutin (*m/z* 743.2) and rutin (*m/z* 610.7) produced an abundant product ion at *m/z* 435.0 and 303.0, respectively. The optimum collision energies were both 22 eV. The product ion mass spectra of troxerutin and rutin are shown in Figs. 1(A) and 1(B). The broken bonds are also marked in Fig. 1.

To optimize the LC system, several columns were tried: $C_{18}$, CN, Phenyl columns. Due to the highly polar character, only a Phenomenex Synergi Fusion RP column achieved suitable retention. In order to achieve maximum peak responses and symmetrical chromatographic peaks, mobile phases containing varying percentages of organic phase were tested. As a result, a mixture of water (containing 0.1% formic acid) and acetonitrile (80:20, v/v) was chosen as the optimized mobile phase.

### Extraction

Troxerutin (see Fig. 1(A)) is a highly polar drug and can hardly be extracted from plasma by organic solvents. Different sample preparation methods (methanol, acetonitrile, acetoacetate and diethyl ether extraction, etc.) were tested and it was found that protein precipitation with perchloric aicd (6%, v/v) was the best. The method was simple, so it can save much time and simplify the operating process. Different volumes of perchloric aicd (6%, v/v) were





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evaluated for efficiency of protein precipitation; it was found that 0.8 times the plasma volume can precipitate the plasma proteins completely, and the chromatographic behavior of the analytes was not deteriorated by this procedure.

## Specificity and matrix effect

The specificity of the method was examined by analyzing blank human plasma (Fig. 2(A)) and spiked with troxerutin (250 pg/mL) and the internal standard (2.5 ng/mL) (Fig. 2(B)). As shown in Fig. 2(A), no significant interferences were seen from endogenous substances in the SRM channels for troxerutin and IS at their corresponding retention times. Figure 2(C) shows the chromatogram for a volunteer plasma sample 1.5 h after an oral dose of 300 mg troxerutin.

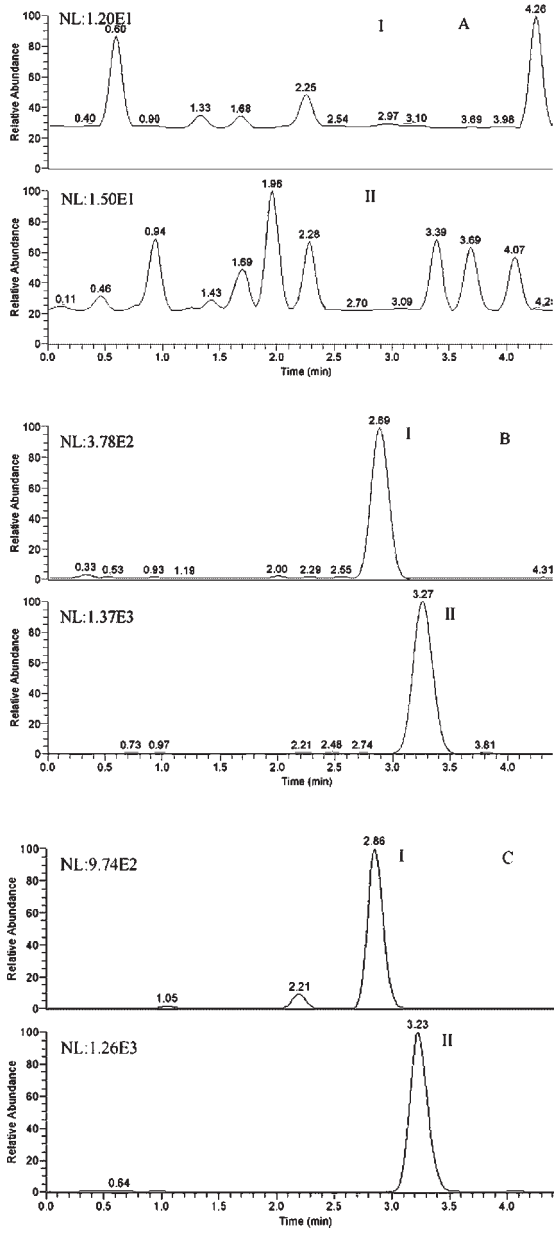


**Figure 2.** SRM chromatograms for troxerutin (I) and rutin (IS, II) in human plasma: (A) blank plasma; (B) blank plasma spiked with troxerutin (250 pg/mL) and the IS (2.5 ng/mL); and (C) a plasma sample from a volunteer 1.5 h after an oral dose of 300 mg troxerutin.

Matrix effects were investigated by analysis of spike-after-extraction samples with pure standard solutions at the same concentrations. The results were 93.4–101.8% for troxerutin and 89.8–103.6% for rutin, and this effect is most likely due to the sample clean-up with perchloric acid.

## Linearity, precision and accuracy

The calibration curves were linear over the concentration range of 31.25–4000 pg/mL for the analyte. Correlation coefficients generated by linear regression with a $1/x^2$ weighting factor ranged from 0.9991 to 0.9996.

The lower limit of quantification (LLOQ) was confirmed to be 25 pg/mL, at which value the calculated accuracy and precision were below 20%.

The intra-day precision and accuracy of the assay were measured by analyzing five spiked samples at each QC level. Inter-day precision and accuracy were accessed over three days. Table 1 summarizes the intra- and inter-day precision and accuracy for troxerutin. In all instances, precision ranged from 4.59–12.28% and relative error was not more than ±12.8%. Both precision and accuracy deviation values were within the acceptable criterion (<±15%), which confirmed the method was accurate and precise enough.

## Recovery and storage stability

The mean extraction recoveries of troxerutin were 114.2, 88.0 and 115.7% at concentrations of 62.5, 250 and 2000 pg/mL, respectively ($n = 3$).

The stability of troxerutin in plasma under different temperature and time conditions was evaluated. The results are given in Table 2. The data indicated reliable stability behavior of troxerutin not only for short-term for at least 12 h, but also after being kept frozen for 1 week. At the same time, the analyte is stable in human plasma for three freeze/thaw cycles. Therefore, no stability problem would be expected during the sample analysis procedure.

**Table 1.** Precision and accuracy for assay of troxerutin in human plasma

| Day of analysis | | Troxerutin concentration in human plasma (pg/mL) | | |
|---|---|---|---|---|
| | | Low QC 62.5 | Medium QC 250 | High QC 2000 |
| | Mean (pg/mL) | 62.46 ± 4.59 | 218.0 ± 23.57 | 2148.2 ± 98.54 |
| Day 1 | RSD (%) | 7.35 | 10.81 | 4.59 |
| | RE (%) | −0.1 | −12.8 | 7.4 |
| | Mean (pg/mL) | 61.18 ± 5.59 | 272.6 ± 22.92 | 1935.2 ± 207.79 |
| Day 2 | RSD (%) | 9.14 | 8.41 | 10.74 |
| | RE (%) | −2.1 | 9.0 | −3.2 |
| | Mean (pg/mL) | 56.68 ± 2.79 | 226.6 ± 20.14 | 2123.0 ± 176.38 |
| Day 3 | RSD (%) | 4.92 | 8.89 | 8.31 |
| | RE (%) | −9.3 | −9.4 | 6.2 |
| Inter-day | SD (pg/mL) | 3.04 | 29.36 | 116.39 |
| Inter-day | Mean (pg/mL) | 60.1 | 239.1 | 2068.8 |
| Inter-day | RSD (%) | 5.05 | 12.28 | 5.63 |
| Inter-day | RE (%) | −3.8 | −4.4 | 3.5 |

RSD: relative standard deviation; RE: relative error.





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**Table 2.** Stability data for assay of troxerutin in human plasma ($n = 3$) under various storage conditions

| Storage conditions | Conc. spiked (pg/mL) | Conc. found (pg/mL) | RSD (%) | RE (%) |
|---|---|---|---|---|
| | 62.5 | 68.6 | 7.25 | 9.8 |
| 4 h at room temperature | 250 | 272.2 | 7.63 | 8.9 |
| | 2000 | 1984.3 | 6.68 | −0.8 |
| | 62.5 | 71.0 | 6.50 | 13.6 |
| 12 h at room temperature | 250 | 278.2 | 5.57 | 11.3 |
| | 2000 | 1968.2 | 0.80 | −1.6 |
| | 62.5 | 54.9 | 9.17 | −12.2 |
| Freezer for 7 days | 250 | 275.4 | 6.83 | 10.2 |
| | 2000 | 2055.1 | 1.92 | 2.8 |
| | 62.5 | 56.2 | 7.44 | −10.1 |
| Three freeze/thaw cycles | 250 | 279.3 | 6.57 | 11.7 |
| | 2000 | 2054.9 | 5.85 | 2.7 |

RSD: relative standard deviation; RE: relative error.

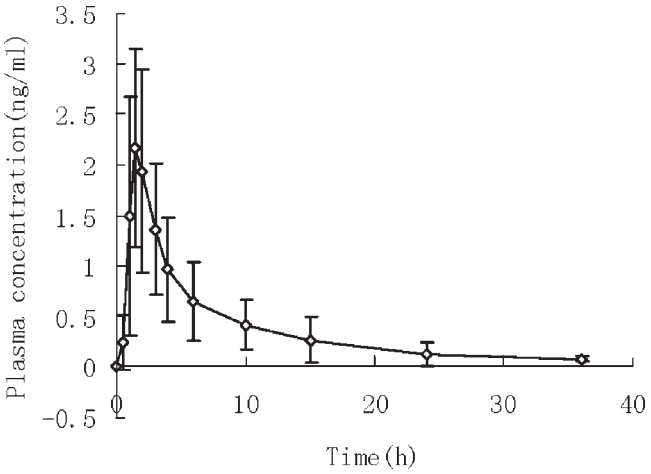


**Figure 3.** Mean plasma concentration time profile for troxerutin after an oral dose of 300 mg troxerutin to 18 healthy volunteers ($n = 18$, mean value and SD are plotted).

## Application to pharmacokinetic study

This validated method was applied to analyze the plasma sample for troxerutin after oral administration of 300 mg troxerutin to 18 volunteers. The profile of the mean plasma concentration versus time is shown in Fig. 3. The maximum plasma concentration ($C_{max}$) was $2931 \pm 1018$ pg/mL; the area under the curve ($AUC_{0-36}$) was $12728 \pm 7092$ pg·h/mL; the time to maximum plasma concentration ($T_{max}$) was $1.44 \pm 0.38$ h; and the half-life ($t_{1/2}$) was $7.94 \pm 3.62$ h. Due to the relatively short chromatographic run time and simple sample preparation procedure, a throughput of 150 samples per day was routinely achieved.

## CONCLUSIONS

We have described a simple, rapid, and sensitive LC/MS/MS assay for the quantification of troxerutin in human plasma, which showed acceptable precision and adequate sensetivity. The method is suitable for the high-throughput analysis of plasma samples obtained in the conduct of pharmacokinetic studies.

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