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Short communication

Oral and i.v. pharmacokinetics of isosteviol in rats as assessed by a new sensitive LC–MS/MS method

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ABSTRACT

A sensitive liquid chromatographic–tandem mass spectrometric (LC–MS/MS) method was developed to investigate isosteviol pharmacokinetics *in vivo*. Isosteviol was extracted from plasma with hexane and 4% formic acid. A Phenomenex Synergi 2μ Fusion reversed phase analytical HPLC column (50 mm × 2.0 mm) equipped with a Synergi 2μ Fusion guard column was employed for chromatographic separations. The gradient mobile phase consisted of acetonitrile (ACN) and 20 mM ammonium acetate at pH 6.5, starting at 20% ACN and ramping to 80% at 7 min, followed by 80% ACN for 1 min, then 20% ACN for 5 min. Negative SRM was used to monitor the *m/z* 317.1/317.1 and 317.3/317.3 transitions for isosteviol and 395.0/395.0 and 397.0/397.0 transitions for internal standard. The retention time of isosteviol was 9.2 min. The assay was linear over the range of 50–2000 ng/mL. The accuracy of the method was in the range of 97–105%. Intra- and inter-day precisions were in the range of 1.5–4.6%. Isosteviol (4 mg/kg) was dosed intravenously and orally to Sprague–Dawley rats ($n = 6$). Plasma samples were collected and analysed. Intravenous isosteviol has a distribution half-life of 35.7 ± 9.0 min with the initial distribution volume of 68.1 ± 9.4 mL. The total clearance, terminal half-life and steady-state volume of distribution were 1.25 ± 0.12 mL/min, 150.6 ± 50.5 min and 272.6 ± 95.9 mL, respectively. The oral bioavailability of isosteviol was found to be 60.4 ± 15.5%.

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1. Introduction

Stevioside (Fig. 1) is a non-caloric sweetener extracted from *Stevia rebaudian*. It possesses anti-hyperglycemic, anti-hypertensive, anti-inflammatory, anti-tumor promoting, anti-oxidant and anti-human rotavirus properties [1]. However, stevioside is metabolized in the gut and absorbed as steviol (Fig. 1) which is mutagenic [2]. Isosteviol (4α, 8β, 13β)-13-methyl-16-oxo-17-norkaurane-18-carboxylic acid, Fig. 1) is a derivative of stevioside which does not possess mutagenic properties.

Isosteviol decreases the blood glucose concentration in Zucker diabetic fatty rats [3] and reduces heart damage induced by ischemia–reperfusion in Sprague–Dawley rats [4]. It also relaxes isolated aortic ring strips in Wistar rats [5] where it is believed to act as a potassium channel opener [6]. In rat aortic smooth muscle cells, isosteviol inhibits angiotensin-II-induced cell proliferation and endothelin-1 secretion [7]. Isosteviol also inhibits activation of tumor promoters in Raji cells [8].

Previous studies using HPLC and LC–MS/MS for the quantitation of isosteviol have been reported but the lower limit of quantitation (LLOQ) were 0.6 and 0.5 μg/mL, respectively [9,10]. These LLOQ may allow preliminary pharmacokinetic studies to be undertaken, but are too high for full characterisation of isosteviol pharmacokinetics at a range of doses. Therefore, in this study, a HPLC LC–MS/MS method has been developed for quantification of isosteviol in rat plasma.

2. Material and methods

2.1. Reagents and chemicals

Isosteviol was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) with purity greater than 99.4%. ZW-r (Fig. 1) was synthesized in-house with purity greater than 98.5%. Formic acid and hexane were obtained from BDH Laboratory Supplies (Poole, England). Acetonitrile (ACN) was purchased from Scharlau Chemie S.A., la Jota (Barcelona, Spain). Ammonium acetate was obtained from Fluka Chemie GmbH (Buchs, Switzerland). Milli-RQ water was prepared by a Milli-RQ Ultrapure Water System (Millipore, USA).

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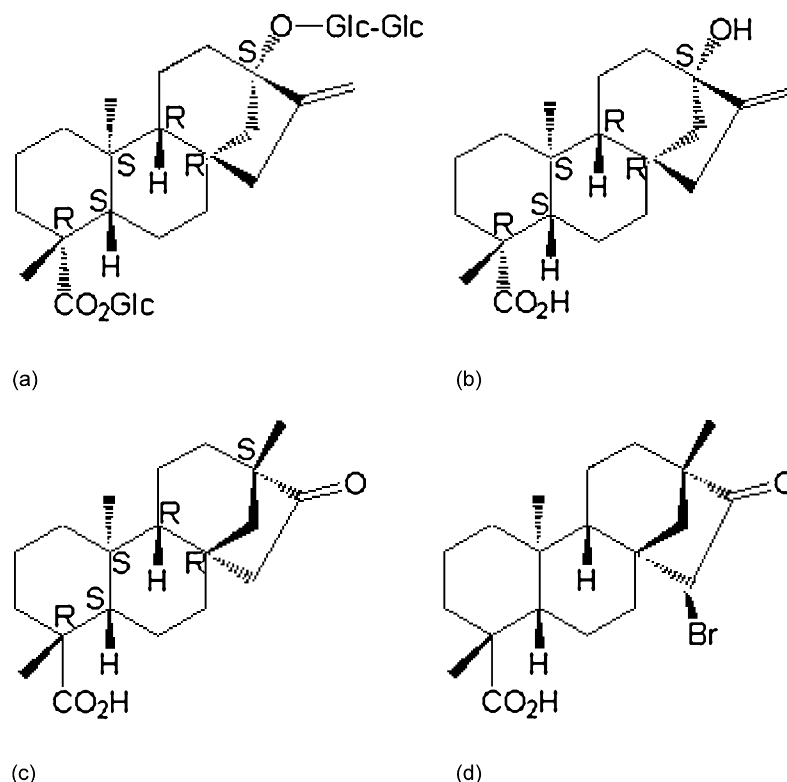

Fig. 1. Chemical structure of stevioside (a), steviol (MW: 318) (b), isosteviol (MW: 318) (c) and internal standard ZW-r (MW: 396) (d). MW, mass weight.

2.2. Animals

Male Sprague–Dawley rats (316 ± 14 g) were purchased from the Animal Resource Centre (Gilles Plains, SA, Australia) and kept in individual cages on a 12 h/12 h light/dark cycle, free access to standard diet food and water in a 20–23 °C room. This study was approved by the Institute of Medical and Veterinary Science (IMVS) Animal Ethics Committee (Adelaide, SA, Australia).

2.3. Sample preparation

Stock solutions of isosteviol calibration standard and quality control were prepared separately in 60 °C water (pH 12.0, adjusted by sodium hydroxide). Calibration standard samples were prepared by spiking the stock solution of isosteviol into heparinised rat plasma to yield final concentrations of 50, 100, 200, 400, 800 1000 and 2000 ng/mL. The blank plasma was obtained from naive Sprague–Dawley rats. Quality control samples were prepared at spiked concentrations of 80, 600 and 1500 ng/mL. The stock solution of internal standard ZW-r (Fig. 1) was prepared in 50% ACN and successively diluted with ACN to result in a final concentration of 1 μg/mL.

Plasma samples (100 μL) were extracted by liquid–liquid extraction. This involved adding 200 μL of 4% formic acid and 1 mL hexane to plasma samples, vortex mixing and centrifugation at 15,000 × *g* for 1 min at room temperature. The supernatant was transferred to a new Eppendorf tube and dried under nitrogen gas at room temperature. The residue was reconstituted in 100 μL 20% ACN and centrifuged at 15,000 × *g* for 1 min at room temperature before transferring to auto-sampler vials. A 20 μL aliquot of each reconstituted sample was analysed by LC–MS/MS.

2.4. LC–MS/MS instrumentation

The sample was injected using an HPLC system consisting of a LC-10AD Shimadzu binary pump and a SIL-HTc Shimadzu auto-sampler (Kyoto, Japan). LC–MS/MS analysis was carried out on an API 3000 mass spectrometer (Applied Biosystems, Foster, Canada). The instrument was operated using atmospheric pressure chemical ionization (APCI). Due to resolution effects, negative SRM was used to monitor the *m*/*z* 317.1/317.1 and 317.3/317.3 transitions for isosteviol with the following instrument settings: declustering potential (DP) −63 V, focusing potential (FP) −200 V, entrance potential (EP) −10 V, collision energy (CE) −17 V and collision cell exit potential (CXP) −9 V. For the internal standard, ZW-r, the instruments settings (V) were DP −52, FP −200, EP −10, CE −15 and CXP −15 to monitor the *m*/*z* 395.0/395.0 and 397.0/397.0 transitions. The needle voltage was −4500 V and the nebuliser temperature was set at 200 °C. Nitrogen was used as nebulising (12 L/min), auxiliary (5 L/min), collision (4 L/min) and curtain (10 L/min) gas. Unit resolution was set for both Q1 and Q3. Dwell time is 100 ms for both isosteviol and the internal standard.

2.5. Chromatographic conditions

A Phenomenex Synergi 2μ Fusion reversed phase analytical HPLC column (50 mm × 2.0 mm) equipped with a Synergi 2μ Fusion guard column was employed for chromatographic separations. The gradient mobile phase consisted of ACN and 20 mM ammonium acetate at pH 6.5, starting at 20% ACN and ramping to 80% at 7 min, followed by 80% ACN for 1 min, then 20% ACN for 5 min as shown in Table 1. The flow rate was 0.2 mL/min.

2.6. Matrix effect and specificity

In order to investigate if there was an issue of ion suppression or ion amplification of the blank matrices, six batches of blank plasma were tested. Blank matrix samples were extracted (as described in Section 2.3) and supplemented with isosteviol at 80, 600 and 1500 ng/mL and internal standard at 1 μg/mL before injecting them directly into the LC–MS/MS system. The ratio of isosteviol and inter-

Table 1
HPLC gradient mobile phase program

Time (min)	Acetonitrile (%)	20 mM NH_4OAc (%)	Flow rate (mL/min)
0	20	80	0.2
8.0	80	20	0.2
9.0	80	20	0.2
10.0	20	80	0.2
13.0	20	80	0.2

nal standard in matrix extract was compared to that in mobile phase.

2.7. Construction of standard curve

Calibration curves were weighted according to $1/x$ (x = concentration) regression. A seven-point calibration curve was constructed using linear regression of the peak area ratios of isosteviol against internal standard ZW-r. Concentrations of isosteviol in plasma samples were calculated using the defined calibration curve equation. The LLOQ was defined as the lowest calibrator with intra- and inter-day precisions (expressed as a percentage of the relative standard deviation, R.S.D.) <20% [11].

2.8. Validation procedure

Six different calibration curves were prepared on different days. The LLOQ was determined by six replicates of the lowest level standard of rat plasma samples. The precision (R.S.D.%) and accuracy of the method were evaluated by determining the QC samples at low, medium and high concentration levels. The accuracy was expressed as a percentage of the calculated concentration to the theoretical concentration.

For determination of the absolute extraction recovery, six replicates of QC samples at 80, 600 and 1500 ng/mL in rat plasma were determined by comparing the peak area of isosteviol in the extracted sample with that obtained from direct injection of the same amount of isosteviol dissolved in mobile phase. Extraction recovery for internal standard was also determined in the similar way.

2.9. Sample dilution

To test whether the method can be applied to samples where the isosteviol concentration is higher than the upper limit of quantitation, spiked QC samples at 20, 10, 5 and 2 μg/mL was prepared and diluted (1:10) with blank matrix in order to bring the concentration within the range of the standard curve then quantified against a calibration curve.

2.10. Stability assay

Stability tests of the analyte were performed on six replicates of three QC concentrations after (a) 8 h at room temperature (short-term stability), (b) three freeze (−20 °C)-and-thaw cycles, (c) reconstituted extract at 4 °C for 24 h and (d) stored at −20 °C for a month, respectively. Concentrations of isosteviol in the stored samples were compared with freshly prepared samples at the same nominal concentration. Stability was not affected by the preparation method of the isosteviol solution.

2.11. Application of the method

Isosteviol plasma concentrations were analysed in a pharmacokinetic study. Rats were divided into two groups (n = 6), cannulated and fasted overnight. They were then dosed with 4 mg/kg isosteviol either orally or intravenously. Blood samples (200 μL) were collected from the jugular vein cannula before dosing and from 5 min to 48 h after dosing. From this, plasma was obtained and stored at −20 °C until analysis. Rats were allowed free access to water throughout and to food from 2 h post-dose.

2.12. Data analysis

WinNonlin 4.1 (Pharsight Corporation, Mountain View, CA, USA) was employed for calculation of isosteviol PK parameters. Non-compartmental analysis was used to determine and compare standard pharmacokinetic parameters in the oral and i.v. treated groups. AUC was calculated using the trapezoidal rule and extrapolated to infinity. Additional parameters relating to the distribution phase observed following intravenous injection were determined with a two-compartmental analysis. Group data are presented as mean ± S.D.

3. Results and discussion

3.1. LC–MS/MS method development

3.1.1. LC–MS/MS conditions

The chemical structure of isosteviol is very similar to steviol (Fig. 1). It is a carboxylic acid, with a mass of 318 amu which lends itself to negative ion detection. After optimizing the APCI source, using unit resolution pure isosteviol gave strong signals for $[C_{20}H_{29}O_3]^-$ at m/z 317.1/317.1 and 317.3/317.3. Both signals of isosteviol were used for detection. Mixtures of ACN and 20 mM NH_4OAc were used as gradient mobile phase. The pH had a strong effect on the intensity of the isosteviol signal, and was optimized at 6.5 to obtain the best response. Gradient elution was employed with the time program is shown in Table 1. Both analytes showed a variation in retention time of less than 1%.

3.1.2. Specificity and matrix effect

Six batches of blank rat plasma were tested with and without analytes. Chromatograms of blank matrix, LLOQ (50 ng/mL) and internal standard (1 μg/mL) are presented in Fig. 2. The retention time of isosteviol was 9.2 min and the internal standard was 10.1 min. There was weak background interference from rat blank plasma (Fig. 2a) coinciding with the isosteviol retention time, but not at the internal standard retention time point. Adequate separation was achieved between isosteviol and the internal standard as shown in Fig. 2d, a representative chromatogram of dosed rat plasma samples. There was no significant interference between isosteviol and internal standard at the retention time.

Matrix effects are generally due to the influence of co-eluting compounds on the actual analyte ionization process. There may be some ion suppression or ion amplification effects from the blank matrices. In the current study, the matrix effect was simply characterised by comparing the ratio of analytes (isoteviol and internal standard) in matrix extracts with that in mobile phase (20% ACN and 20 mM ammonium acetate). As shown in Table 2, there was

Table 2
Matrix effect on LC–MS/MS assay for isosteviol (n = 6; mean ± S.D.)

Nominal concentration (ng/mL)	In mobile phase[a]	In plasma extract[b]
80	0.191 ± 0.007	0.193 ± 0.006
600	1.226 ± 0.020	1.229 ± 0.027
1500	2.680 ± 0.026	2.669 ± 0.141

[a] The peak area ratio of isosteviol and internal standard in mobile phase.
[b] The peak area ratio of isosteviol and internal standard in plasma extract.

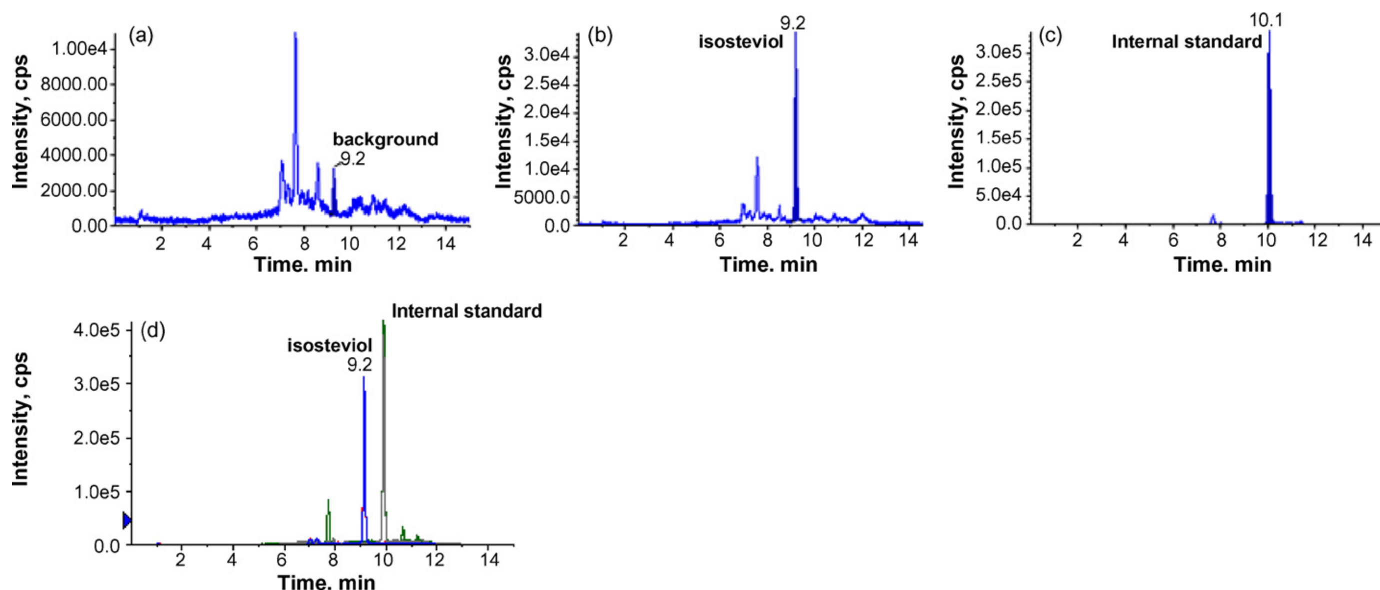

Fig. 2. LC–MS/MS chromatograms of isosteviol, internal standard and matrix. (a) Chromatogram of blank plasma, there is a small peak at isosteviol retention time. (b) Chromatogram of internal standard ZW-r (1 μg/mL) only. (c) Chromatogram of isosteviol at LLOQ (50 ng/mL). (d) A representative chromatogram of rat plasma samples (239 ng/mL), 8 h after oral dosing isosteviol (4 mg/kg).

no significant ion effect of the blank matrix on the peak area of isosteviol at different concentrations.

3.1.3. Calibration curve

The linearity of the assay was achieved over the range of 50–2000 ng/mL with coefficients of correlation (r) greater than 0.998. The equation of the six standard curve was expressed as $y = (0.0243 \pm 0.0030)x - (0.0549 \pm 0.0057)$ with $1/x$ weighting regression. The precision (R.S.D.%) and accuracy (percentage of the calculated concentration relative to nominal concentration) of the calibration standards of six batches are shown in Table 3. The accuracy of each standard was between 98 and 104%. The LLOQ of the assay was determined by six replicates at the lowest concentration of the standard curve: 50 ng/mL.

3.1.4. Validation

Intra-day precision and accuracy were assessed by six replicates of each of the three QC concentrations (Table 4). The intra-day precision (R.S.D.%) was within 2.0%, while the intra-day accuracy (expressed as percentage of nominal values) ranged from 97 to 102%. The inter-day precision and accuracy were determined by each QC concentration in each of the six assay runs ($n = 16$; Table 4). The inter-day precision (R.S.D.%) was within 4.6% and accuracy was in the range of 97–103% (Table 4).

Samples with concentrations higher than the upper limit of quantitation were diluted into the linear range with blank plasma. The results of dilution for QC samples spiked at 20, 10, 5 and 2 μg/mL (Table 4) show that samples with high isosteviol concentrations can be successfully diluted into the linear range.

Table 3
Back calculated isosteviol concentrations and their relative standard deviation (R.S.D.) and accuracy for calibration curves performed in rat plasma ($n = 6$)

Nominal concentration (ng/mL)	Observed concentration (ng/mL; mean)	R.S.D. (%)	Accuracy (%)
50	49	4.2	99
100	99	3.9	99
200	195	4.0	98
400	418	1.2	104
800	820	2.4	103
1000	982	4.1	98
2000	1990	1.8	99

3.1.5. Recovery

The absolute recoveries of isosteviol from plasma QC samples at three concentrations were 76 ± 3% for 80 ng/mL; 73 ± 2% for 600 ng/mL; 76 ± 1% for 1500 ng/mL and 60 ± 4% for the internal standard. The variation (CV) of the extraction recovery for both isosteviol and the internal standard was less than 7.3%.

3.1.6. Stability

The stability of isosteviol in rat plasma was investigated at three QC levels, as described in Section 2.9. The difference between the tested samples and fresh prepared samples at three QC concentrations were less than 5%. The results showed that isosteviol was stable under all the tested conditions. At three QC concentrations, sample diluted from stock solution prepared in 60 °C H_2O had no

Table 4
Accuracy and precision of the LC–MS/MS assay for isosteviol in rat plasma

Nominal concentration (ng/mL)	Reported concentration (ng/mL)	Precision (R.S.D.%)	Accuracy (%)
Intra-day ($n = 6$)			
80	81 ± 1	1.5	101
600	614 ± 12	1.9	102
1,500	1,450 ± 30	2.0	97
Inter-day ($n = 16$)			
80	79 ± 4	4.6	99
600	617 ± 19	3.1	103
1,500	1,454 ± 46	3.2	97
[a] Dilution control ($n = 6$)			
2,000	2,097 ± 76	3.6	105
5,000	5,090 ± 104	2.1	102
10,000	10,083 ± 203	2.0	101
20,000	19,933 ± 339	1.7	100

[a] Dilution controls were applied to test if the standard curve is suitable for measuring samples whose concentration is higher than the upper limitation. Plasma aliquot was spiked to a nominal concentration which was diluted 1 in 10 for dilution QC samples. The sample was measured which allowed calculation of the reported concentration for dilution QC samples.

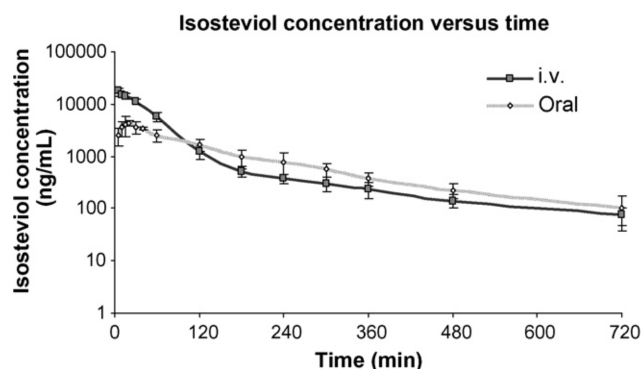

Fig. 3. Concentrations (mean ± S.D., $n = 6$) of isosteviol in rat plasma versus time after i.v. or oral administration (4 mg/kg).

Table 5
Pharmacokinetic parameters of isosteviol in rats *in vivo* ($n = 6$)

i.v.	
AUC_{inf} (min µg/mL)	1046.2 ± 62.7
$t_{1/2}$ (min)	150.6 ± 50.5
CL (mL/min)	1.25 ± 0.12
V_Z (mL)	272.6 ± 95.9
V_{ss} (mL)	88.7 ± 11.4
C_0 (µg/mL)	20.2 ± 5.3
Oral	
AUC_{inf} (min µg/mL)	632.3 ± 162.0
$t_{1/2}$ (min)	169.9 ± 46.6
C_{max} (µg/mL)	4.24 ± 1.31
t_{max} (min)	10–15
F (%)	60.4 ± 15.5

AUC_{inf}, area under the analyte concentrations versus time curve from time 0 to infinity; $t_{1/2}$, terminal half-life; CL, clearance; V_Z, terminal volume of distribution; V_{ss}, volume of distribution at steady state; C_0, when time = 0, analyte concentration; C_{max}, maximum concentration; t_{max}, the time of maximum concentration; F, bioavailability.

significant differences from samples diluted from isosteviol stock solution (2 mg/mL) in ACN. The stock solution of isosteviol was stable for a month at −20 °C.

3.2. Method application

The isosteviol concentration in plasma versus time profile is shown in Fig. 3. From the profile, it is apparent that isosteviol concentrations decline in a bi-exponential manner after i.v. administration. The half-life of initial distribution phase is 35.7 ± 9.0 min with an initial distribution volume of 68.1 ± 9.4 mL. Other pharmacokinetic parameters are provided in Table 5. The low LLOQ provided by the analytical method enabled the pharmacokinetics of isosteviol to be characterised much better than when using previous analytical methods. In particular, we were able to observe the terminal phase of the plasma concentration–time curve which may have been missed at this dose using previous methods. It also enabled an accurate estimation of oral bioavailability. To the best of our knowledge, this is the first study to report the oral pharmacokinetics of isosteviol.

4. Conclusions

This developed method has been shown to be reproducible, reliable and sensitive. It has been used to characterise isosteviol PK properties in rats and proved sensitive enough for detecting isosteviol for at least 12 h after dosing (4 mg/kg). This is the first reported characterisation of oral isosteviol pharmacokinetics.

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