Determination of isosteviol by LC-MS/MS and its application for evaluation of pharmacokinetics of isosteviol in rat

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ABSTRACT

Isosteviol has been found to have potential preventive or therapeutic effects against hypertension, ischemia reperfusion injury, diabetes and cancer, but little is known about the pharmacokinetics (PK) of the compound. The aim of this study was to develop a liquid chromatographic-tandem mass spectrometric (LC-MS/MS) method for determination of isosteviol in rat plasma and to assess in a preliminary manner the PK of isosteviol after intravenous bolus injection.

Ions of analytes were generated using electro-spray ionization and detected in the positive-ion mode in LC-MS/MS. Multiple reaction monitoring was performed, using the precursor product ion combination for isosteviol m/z 319.4→273.4. Progesterone was used as an internal standard. Nitrogen was used as the nebulising gas and unit resolution was set for Q1 and Q3. Isosteviol solution was injected through the penile vein of rats at a dose of 8 mg/kg. Blood samples were collected from a jugular vein cannula. The PK parameters were calculated using a two - compartment PK model.

The LC-MS/MS assay for isosteviol in rat plasma was linear over the range of 0.5-80 μg/ml. The terminal half life of isosteviol ($t_{1/2}$) was 406 ± 31.7 min and clearance (CL) was 2.9 ± 0.3 ml/min/kg. A sensitive LC-MS/MS assay for isosteviol in plasma has been successfully established and used in a preliminary PK evaluation of isosteviol in rats.

**Keywords:** Isosteviol, LC-MS/MS, Pharmacokinetics, Rats

INTRODUCTION

Isosteviol (4α,8β,13β-13-methyl-16-oxo-17-nor-kauran-18-oic acid), a derivative of stevioside (Figure 1) which is a sweet-tasting glycoside present in the leaves of the herb S*tevia rebaudiana*, has been used widely in Japan and Brazil as a sugar substitute (1). It tastes about 300 times sweeter than sucrose (0.4% solution) and has recently attracted much attention as an alternative sweetener (2-4).
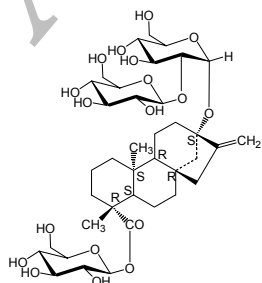
Figure 1. Chemical structure of stevioside

Hydrolysis of stevioside in acid solution produces isosteviol, a tetracyclic diterpenoid with a beyerane skeleton (Figure 2) (5).

The biological activities of isosteviol include insect anti-feeding actions, inhibition of rat liver mitochondria function (5-7), a blood pressure lowering effect (3,8), a capacity to decrease glucose production and to inhibit oxygen uptake in the isolated rat renal tubules (9), as well as vasorelaxatory and cardioprotective effects (10,11).
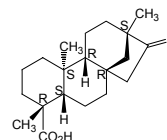
Figure 2. Chemical structure of isosteviol

Also it has been shown that isosteviol potently inhibited both mammalian DNA polymerase and human DNA topoisomerase Π and it prevented the growth of human cancer cells (12). The compound did not induce mutagenesis (7,13) and could be considered a possible candidate for treatment of cancer (13,14).

Despite the growing pharmacological interest in isosteviol, there is no report on the pharmacokinetics of the compound in animals or

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humans. In order to perform pharmacokinetic studies, a sensitive and specific analytical method is needed. The aim of this study was therefore to develop a sensitive and specific method for determination of isosteviol in plasma by LC-MS/MS and to determine its preliminary pharmacokinetic properties after IV bolus injection.

MATERIAL AND METHODS

Animals

Male Sprague-Dawley rats (350-400 g) were obtained from the Institute of Medical and Veterinary Science (IMVS) Animal Resource Centre, Gilles Plains, South Australia. The study was approved by the IMVS animal ethic committee. The right jugular vein of each animal was cannulated 12-16 hours before dosing. For surgery, rats were induced to anesthetic status with halothane/oxygen vapor (4%) set at a flow rate of 1.5 l/min; and then anesthetic status was maintained by continuous administration of halothane/oxygen mixture (2%) at a flow rate of 600 ml/min throughout surgery. A longitudinal incision of approximately 2-3 cm in the skin over the jugular vein was made. By exposing and making a small incision in the vein, a cannula (silicone tubing 0.51 mm ID ×0.94 mm OD, 3.5-4 cm in length, with a vinyl tubing extension 0.50 mm ID × 1 mm OD) was inserted and threaded towards the heart (3-5 cm) and fixed by suturing. The cannula was filled with heparanized saline and passed through the skin of the rat between the shoulders and a map pin was inserted to block the end. Rats were fasted after surgery (but had free access to water) and retained in metabolic cages during the experiment.

Materials

Isosteviol was a gift from Prof. Deyi Xu from Southeast University, China. Acetonitrile, acetic acid and methanol, all HPLC grade, were purchased from BDH Laboratory Supplies (Poole, UK). Progesterone was supplied by Sigma-Aldrich (St. Louis, MO, USA).

Water was deionized, filtered and purified by an Ultra Pure Water System (Millipore, South Australia).

Administration of isosteviol and sample collection

Isosteviol was dissolved in a mixture of methanol, propylene glycol and saline, (20:20:80, v/v/v, pH 7.4) for IV injection. The compound was injected through the penile vein of the rats (n=5) at a dose of 8 mg/kg body weight. For dosing, rats were anaesthetized and the compound was injected with a 23 G needle (Livingstone International Pty Ltd, Rosebery, NSW) slowly over 30 seconds. Blood samples (0.25 ml) were collected from the jugular vein cannula before dosing (immediately prior to isosteviol administration) and 5, 10, 15, 20, 30, 40, 60, 90 min 2, 3, 4, 6, 8, 10, 24 and 48 hours after dosing. Rats had free access to food and water after 2 hours of sampling. The blood samples were transferred to heparinized eppendorf tubes (Sarstedt Australia Pty Ltd, Technology Park, South Australia) and centrifuged at 5000 rpm for 5 min. Plasma was removed and frozen at -20°C until analysis. After collection of each blood sample, the cannula was flushed with 0.25 ml of heparinized saline.

Urine was collected at intervals of 0-4, 4-8 and 8-24 hrs after dosing into previously weighed containers and samples retained at -20°C until analysis.

Determination of isosteviol in rat plasma and urine by LC-MS/MS

Plasma samples had to be treated in acidic medium to prevent adsorption of the drug onto the precipitated protein. To 100 µl of plasma, was added 10 µl of progesterone (50 µg/ml) as internal standard. The mixture was treated with 400 µl of acetonitrile:acetic acid (95:5 v/v). The samples were vortex mixed for 1 min, centrifuged at 5000 rpm for 10 min and the supernatant (10 µl) injected into the LC-MS/MS system (API 3000, Applied Biosystems, Canada).

Urine samples were centrifuged, diluted with water (1/10) and 10 µl injected onto the LC-MS/MS. The methodology was validated by spiking rat urine samples with known amounts of isosteviol.

The plasma and urinary concentrations of isosteviol was determined using the conditions described below.

A C18 guard column was used for partial separation of compounds. Isosteviol eluted at 1.5 min and progesterone at 1.55 min, with a total run time of 4 min. Ions were generated using electrospray ionization and detected in the positive-ion mode. The mobile phase consisted of acetonitrile:water:acetic acid (80:19:1 v/v/v), delivered by a Shimadzu LC-10AD solvent delivery system at a rate of 0.2 ml/min. A sample volume of 10 µl was injected using a Shimadzu SIL-HTc autosampler.

Multiple reactions monitoring was performed using the precursor-product ion combination for isosteviol m/z 319.4→273.4 at instrument settings of: DP.101, FP.350, EP.10, CF.23, and CXP.19.

Progesterone was monitored (m/z 315→97 ion pair) at instrument settings of DP.83, FP.350, EP.10, CE.35, and CXP.6. Ultra-high purity

nitrogen was used both as the nebulising (12 l/h), auxiliary (5 l/h), collision (4 l/h) and curtain (10 l/h) gas. Unit resolution was set for Q_1 and Q_3. Calibration samples consisted of 7 different concentrations of isosteviol over the range of 0.5 to 80 µg/ml. These were prepared by adding a stock solution of methanolic isosteviol (4 mg/ml) with human plasma. The low, medium and high quality control samples (0.8, 16, 48 µg/ml) were prepared independently in human plasma. Calibration lines were prepared using $1/y*y$ weighting.

Pharmacokinetics of isosteviol
The time course of isosteviol concentration in plasma was evaluated by two- compartment pharmacokinetic analysis using WinNonlin software package (Pharsight Corporation, Mountain View, CA, USA) and the followings were calculated using standard methods: the area under plasma isosteviol concentration versus time (from zero to infinity) curve, AUC (0-∞); elimination rate constant, k_{el}; half-life, $t_{1/2}$; clearance, CL and volume of distribution, Vd.

RESULTS AND DISCUSSION
An LC-MS/MS assay for isosteviol in rat plasma was successfully established and used to evaluate the preliminary PK study of isosteviol in rats. There was clear separation between the analyte and internal standard (Figure 3).
The LC-MS/MS assay for isosteviol was linear over the range of 0.5-80 µg/ml with a correlation coefficient of 0.996. The method had a lower limit of quantification (LLOQ) of 0.5 µg/ml for isosteviol (Figure 4).
The intra-day and inter-day precision and accuracy for repeated analyses of quality control samples are tabulated in Table 1. The inter-day and intra-day accuracy and precision were all within 10%, with inter-day reproducibility assessed on 3 different days.
After bolus intravenous administration of isosteviol, its concentration in plasma declined in a bi-exponential manner (Figure 5) with an initial phase lasting for about 150 minutes followed by a much slower decline in plasma levels. The pharmacokinetic parameters obtained from the concentration curves versus time are reported in Table 2, as well as described below.
For isosteviol, the volume of distribution (Vd) was 1689 ± 73.1 ml, clearance (CL) was 2.9 ± 0.3 ml/min/kg and the terminal half life ($t_{1/2}$) was 406 ± 31.7 min. The volume of distribution of isosteviol was much higher than the volume of rat plasma (typically 10-13 ml) which indicates extensive distribution outside of plasma. The extensive distribution and relatively low clearance led to a very long terminal half life.
Analysis of urine samples collected from 0.01 to 4 hr after dosing did not reveal any trace of isosteviol, suggesting negligible renal excretion of unchanged drug. The maximum amount of isosteviol excreted in urine up to 4 hours after dosing was only 2.58 µg (LLOQ x Urine volume) which is approximately 0.09% of the dose.

**Table 1.** Intra-day and inter-day assay reproducibility for determination of isosteviol

Concentration (µg/ml)	Measured (µg/ml) Mean ± SD, n=6	C.V.%	Diff. %
Intra-day assay			
0.80	0.82 ± 0.06	7.30	2.3
16.00	17.1 ± 0.69	4.05	6.2
48.00	50.2 ± 4.67	9.31	4.4
Inter-day assay(3 days)			
0.80	0.793 ± 0.075	9.6	-0.8
16.00	16.9 ± 0.81	4.8	3.3
48.00	49.6 ± 3.98	7.9	3.2

Data presented as mean with standard deviation (SD); CV%, coefficient of variation; Diff %, different from actual concentration.

Table 2. Pharmacokinetic parameters of isosteviol in rat plasma after intravenous bolus injection (8 mg/kg).

| PK parameter	Mean	SD	CV%
α (min^{-1})	0.011	0.0007	6.39
β (min^{-1})	0.0017	0.00015	8.57
$T_{1/2}$ (min)	406	31.7	7.81
AUC(0-inf) (µg/min/ml)	1273	79	6.2
Vd (ml)	1689	73.1	4.33
CL (ml/min/kg)	2.9	0.3	10.4

Group data from five rats are presented as mean ± standard deviation (SD). CV, Coefficient of variation; α, Distribution constant; β, Elimination constant; $T_{1/2}$, Biological half life; AUC, Area under curve; Vd, Volume of distribution; CL, Clearance
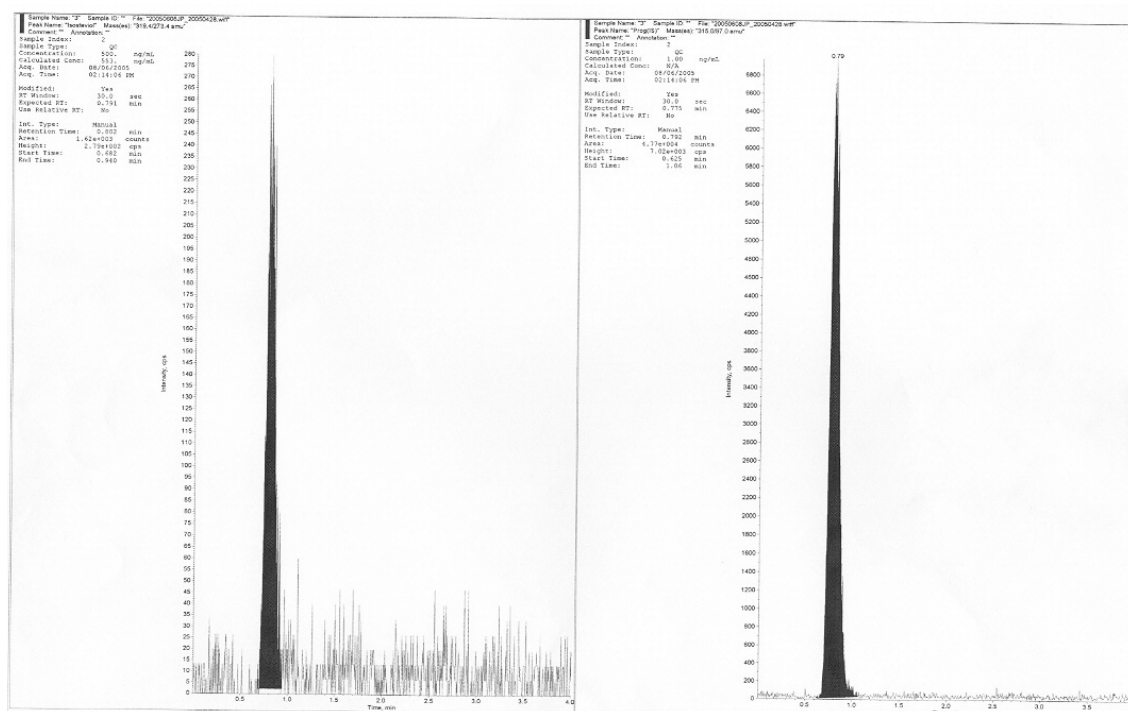
Figure 3. LC-MS/MS chromatograms of isosteviol (left, 0.5 µg/ml) and progesterone (right) for a standard calibration curve sample.

$$y = 0.0354x + 0.0327 \quad (R^2 = 0.9961)$$
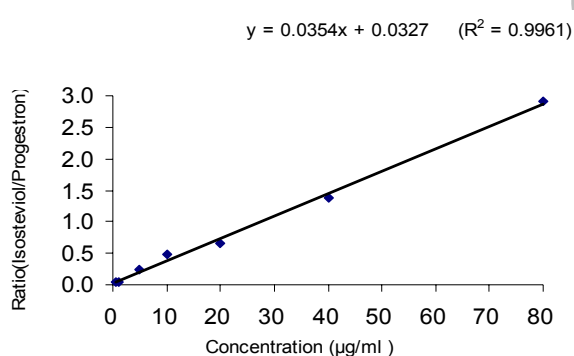
Figure 4. Calibration curve of isosteviol
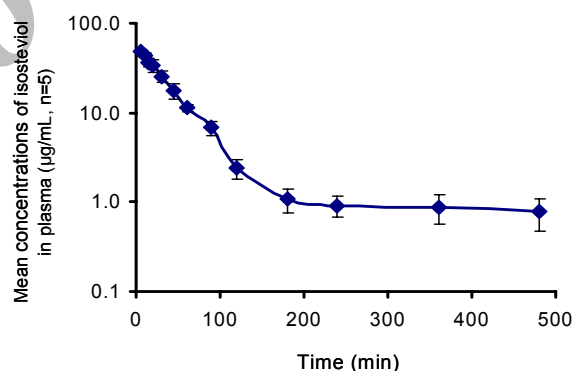
Figure 5. Concentration of isosteviol in rat plasma (Mean $\pm$ SD) versus time profile after an intravenous bolus dose (8 mg/kg) to 5 rats

The described method allows reliable and accurate determination of isosteviol in biological samples. With growing interest in the pharmacological activity of isosteviol, the needs for a comprehensive evaluation of its pharmacokinetics is becoming necessary, and further studies will evaluate the impact of dose size on the pharmacokinetics of this intriguing compound.

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