



Erythrocyte Partitioning Profile of Isosteviol in Human and Rat Blood

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ABSTRACT

Background: Isosteviol is a synthetic derivative of steviol glycosides with promising pharmacological properties and might find future use as a cardioprotective agent.

Objective: A simple LC-MS/MS technique was developed and validated for the bioanalysis of isosteviol in plasma and erythrocytes. This method was subsequently utilized for the in vitro assessment of isosteviol's partitioning into blood compartments of humans and rats.

Methods: Fresh blood samples from healthy humans and Wistar rats were equilibrated with 1, 10, and 30 μ M isosteviol at 37 °C in a shaking dry-bath. The levels of isosteviol in plasma and erythrocytes partitions were determined in these samples, after separation, at intervals over a 60-minute period. The data derived was used to estimate erythrocyte-to-plasma and blood-to-plasma coefficients.

Results: Mean erythrocyte-to-plasma partition coefficients (SD) after 60 minutes of equilibration were observed to be 0.039 (0.002) and 0.040 (0.003) in humans and rats, respectively. Derived values for the blood-to-plasma ratio (SD) were 0.576 (0.001) in humans and 0.543 (0.007) in rats, whereas plasma component binding was estimated to be more than 96%.

Conclusions: The findings suggest that isosteviol preferentially partitions into plasma compartments in humans and rats. The significance of this profile for the efficacy, tissue uptake, and retention of isosteviol will have to be further studied.

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Introduction

Isosteviol is a synthetic derivative of steviol glycosides that has been the focus of several studies in recent times owing to pharmacologic effects associated with it. Neuroprotective attributes in cerebral ischemia model¹ and the attenuation of action potential in hypertrophied cardiomyocytes² are some major findings of potential clinical significance that have been reported recently. Although these properties have also been associated with steviol glycosides,³ a combination of poor intestinal absorption, extensive metabolism,⁴ and a much-debated genotoxicity⁵ limits the therapeutic usefulness of isosteviol's glycosides. Preliminary studies with isosteviol suggest promising benefits because of its

reasonable absorption⁶ with no documented genotoxicity issues previously associated with steviol glycosides.²

A pharmacokinetic study of isosteviol in rats, utilizing plasma, by Jin et al⁶ observed that a mean peak level of 4.24 μ g/mL ($\sim$ 13 μ M) was reached within 15 minutes of oral administration of a 4 mg/kg dose. Mean half-life values were also reported to be 150.6 minutes and 169.9 minutes after single intravenous and oral doses, respectively. Although plasma is a popular biological fluid of choice for pharmacokinetic sample analysis, the erythrocyte-partitioning and plasma-protein-binding properties of drug molecules help provide better physiological description of plasma-derived pharmacokinetic parameters.⁷ A better understanding of the disposition of isosteviol would benefit immensely from a clearer understanding of its interaction with blood components. In addition, data on isosteviol's partitioning into erythrocytes will serve to reinforce or weaken considerations for potential hematotoxicity in the course of its development as a therapeutic candidate in future.

This study, therefore, investigated the distribution of isosteviol in blood compartments to provide parameters describing its

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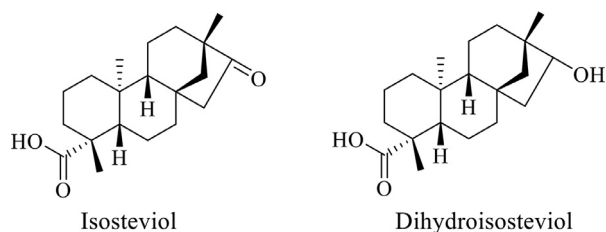


Figure 1. Chemical structures of isosteviol and the internal standard, dihydroisosteviol.

erythrocyte-partitioning and plasma–protein-binding potential in humans and a preclinical animal.

Materials and Methods

Sample preparation

Stock solutions of isosteviol (Figure 1) (purchased from Sigma, Steinheim, Germany) were prepared in dimethyl sulfoxide. The internal standard, dihydroisosteviol (Figure 1), was synthesized in-house and its stock solution was prepared in LC-MS grade methanol.

Whole blood samples were drawn from 6 healthy human volunteers, and from healthy Wistar rats ($n=6$). Plasma and erythrocytes of humans and rats were separated by centrifugation of whole blood collected in K_2 -EDTA bottles at 9391 g for 10 minutes. These matrices were used for the preparation of calibration curves in the range of 0.05 μ M to 25 μ M for isosteviol. The cellular and plasma fractions were spiked accordingly and vortexed for 1 minute. One hundred microlitre aliquots of spiked plasma samples were protein-precipitated with 400 μ L ice-cold methanol (MH) containing 0.3 μ M dihydroisosteviol (internal standard). The mixture was briefly vortexed for 1 minute and thereafter centrifuged at 9391 g for 10 minutes to separate the clear, protein-free, supernatant for LC-MS/MS analysis. Further, spiked erythrocytes were lysed by storage at -80°C for 24 hours followed by a return to room temperature before a similar protein precipitation with cold MH spiked with the internal standard. Vortex mixing and sample centrifugation was similarly carried out before further analysis.

Erythrocyte partitioning of isosteviol was determined in fresh human or rat blood samples. Packed cell volume (44% and 48% in human and rat samples, respectively), used as a surrogate for hematocrit values of these samples, was determined by centrifugation. Whole blood samples were preincubated for 30 minutes at 37°C before being spiked with isosteviol to final concentrations of 1 μ M, 10 μ M, and 30 μ M in triplicate, in 1 mL as final volume. These samples were further kept at 37°C in a shaking dry-bath (Allsheng, Zhejiang, China). Subsequently, aliquots were sampled for centrifugation at 9391 g for 10 minutes after 0, 15, 30, 45, and 60 minutes of incubation with shaking. Plasma and erythrocyte samples were treated as described earlier before analysis. Extracts from incubation at 30 μ M isosteviol were diluted 1:3 with distilled water before analysis. Analytes recovered from the incubation of erythrocytes with 1 μ M isosteviol were concentrated to 4 times their initial concentration under vacuum at 25°C .

LC-MS/MS analysis

Isosteviol in human and rat samples was analyzed with a Xevo TQ-S mass spectrometer (Waters; Milford, Massachusetts) connected to an ACQUITY UPLC system (Waters) via an electrospray ionization interface. Chromatographic separation of analytes was done with an ACQUITY BEH C18 column (Waters) (3.0×50 mm i.d.; 1.7 μ m particle size) at 40°C with samples injection from an

autosampler at 15°C . The mobile phase comprised 5 mM ammonium acetate (AA) and MH pumped through the column in a gradient over a 10-minute period after a 5 μ L sample injection. Analyte resolution was achieved by initially pumping a 50:50 ratio of AA:MH through the column for 4 minutes followed by a switch to 25:75 ratio of AA:MH for 2 minutes. The mobile phase was then switched to a 10:90 ratio of AA:MH for 1 minute and then returned to the initial ratio of 50:50 for another 3 minutes.

Data acquisition from the Xevo T-QS mass spectrometer was done in the negative mode to monitor a transition of m/z 317.21 $\rightarrow$ 273.22 (cone voltage 20 V, collision energy 28 eV) for isosteviol. A transition of m/z 319.21 $\rightarrow$ 319.21 (cone voltage 30 V, collision energy 15 eV) was monitored for the internal standard as a suitable fragment could not be found. Source and desolvation temperatures were set at 150°C and 500°C , respectively. A further scan of samples in the MS1 mode was carried out to detect the likely production of metabolites of isosteviol.

Data analysis

The partition coefficient of isosteviol in erythrocyte, $K_{e/p}$, was calculated as a ratio of the concentration of isosteviol in the erythrocyte fraction (C_e) to that of its concentration in the plasma fraction (C_p) (Equation 1). Whole blood-to-plasma concentration ratio, $K_{b/p}$, was thereafter calculated using the relationship expressed in Equation 2 where H_c is the hematocrit. Furthermore, it was assumed that only protein-free, unbound, isosteviol molecules could interact/partition into erythrocytes. Hence, the degree of plasma compartment binding in 60 minutes of incubation ($f_{b,60 \text{ min}}$) was estimated from the whole blood-to-plasma ratio, and haematocrit using the relation expressed in Equation 3^{7,8} with C_p and C_b being the concentrations of isosteviol in plasma and blood, respectively.

$$K_{e/p} = C_e/C_p \quad (1)$$

$$K_{b/p} = K_{e/p} \cdot H_c + (1 - H_c) \quad (2)$$

$$f_{b,60 \text{ min}} = 1 - \left[\left(f_{u,p \ 60 \text{ min}} \cdot C_p/C_b \right) \times (1 - H_c) \right] \quad (3)$$

Results

The assay parameters are summarized in Table 1. Isosteviol was eluted at 6.34 minutes, while the internal standard has a retention time of 6.66 minutes. The limits of detection were 0.056 μ M and 0.055 μ M in human and rat plasma, respectively, with corresponding quantitation limits of 0.171 μ M and 0.168 μ M. In erythrocytes, the detection limits observed were 0.043 μ M and 0.048 μ M in human and rat samples, respectively, with corresponding assay quantitation limits of 0.129 μ M and 0.145 μ M. Back-calculated concentrations of calibration standards for plasma and erythrocyte samples were all less than $\pm 15\%$ of corresponding nominal values. An MS1 scan of isosteviol samples after 1-hour incubation in fresh blood showed that no detectable metabolite was formed. Data acquired for the partitioning of isosteviol into blood compartments is summarized in Table 2. In human blood, $K_{e/p}$ values (%CV) of 0.037 (14.54), 0.038 (7.97), and 0.034 (4.69) were observed after 60 minutes of 1 μ M, 10 μ M, and 30 μ M isosteviol incubation, respectively. For rats, $K_{e/p}$ (%CV) were 0.046 (48.71) and 0.047 (44.41) at 1 μ M and 10 μ M isosteviol concentrations, respectively, after 60 minutes of incubation at 37°C .

Discussion

The model adopted in this study provides information on the extent of binding or uptake of isosteviol into cells, and perhaps

Table 1

Summary data regarding analysis of isosteviol in biological matrices.

Human	Isosteviol concentration			
	0.25 μM	1.5 μM	5 μM	25 μM
Plasma				
Within-run imprecision	2.04	3.32	0.24	1.25
Between-run imprecision	2.52	7.03	5.51	3.01
Absolute recovery in % [CV %]	89.54 [2.41]	88.77 [1.97]	80.33 [5.14]	86.55 [3.55]
Erythrocytes				
Within-run imprecision	3.96	4.29	0.42	0.72
Between-run imprecision	6.21	12.85	2.98	2.17
Absolute Recovery in % [CV %]	97.31 [3.92]	102.32 [2.69]	93.88 [0.64]	77.59 [4.01]
Rat				
Plasma				
Within-run imprecision	4.01	4.63	1.94	0.64
Between-run imprecision	7.96	7.34	5.20	4.28
Absolute Recovery in % [CV %]	91.16 [3.61]	87.65 [2.73]	88.77 [2.62]	88.13 [1.50]
Erythrocytes				
Within-run imprecision	4.06	5.20	1.77	1.11
Between-run imprecision	8.26	8.42	1.49	3.12
Absolute Recovery in % [CV %]	88.79 [3.98]	86.50 [2.51]	80.82 [2.44]	89.31 [1.62]

All parameters were generated from 6 replicate samples; CV: coefficient of variation.

Table 2

Summary data of erythrocyte partitioning of isosteviol in human and rat blood.

Concentration, μM	Mean for 0–60 min (%CV)		
	$K_{e/p}$	$K_{b/p}$	$f_{b,60 \text{ min}}$
Human			
1	0.037 (14.54)	0.576 (0.23)	0.971 (0.27)
10	0.038 (7.97)	0.576 (0.21)	0.950 (0.42)
30	0.034 (4.69)	0.575 (0.12)	0.966 (0.30)
Rat			
1	0.046 (48.71)	0.542 (2.00)	0.972 (1.24)
10	0.047 (44.41)	0.543 (1.87)	0.963 (1.79)
30	0.072 (33.75)*	0.555 (2.10)	0.947 (1.92)

$f_{b,60 \text{ min}}$ = estimated fraction of isosteviol bound to plasma compartments in 60 minutes. $K_{b/p}$ = whole blood-to-plasma ratio; $K_{e/p}$ = erythrocyte partition coefficient.

* Unusual change likely due to saturation at plasma-binding-sites in rat blood.

tissues, using the erythrocyte as a reference compartment. The results obtained suggests that isosteviol preferentially interacts with human plasma compartments than those of erythrocytes as shown by erythrocyte-to-plasma partition coefficient values of less than 1.0 at all concentrations studied. It is logical to assume that this could only have arisen from a significant binding of isosteviol to plasma proteins that constitute a significant portion of the plasma compartment.

Estimated mean (SD) values of bound isosteviol molecules after 60 minutes of incubation, $f_{b,60 \text{ min}}$ (Table 2) in human blood was observed to be 0.96 (0.01), and in a manner that was independent of concentration in the range studied. The estimated high binding of isosteviol in human plasma may not necessarily correspond to similar behavior in human tissues as shown with certain drugs like imipramine.⁹ A greatly reduced unbound concentration of isosteviol in the blood as predicted from this calculation could portend reduced efficiency of its removal by glomerular filtration and other kidney transport systems, and a reduced overall rate of elimination in humans.¹⁰ This assumption precludes the possibility of an efficient hepatic biotransformation whose generation of polar metabolites might lead to an increased elimination rate of isosteviol should $f_{b,60 \text{ min}}$ represent the true value of the amount of isosteviol bound to plasma protein.

Considering the acidic nature of isosteviol and the tendency that it exists in its anionic form at physiological pH, human serum albumin known for its preference for similar anionic drugs¹¹

seems the most probable protein of interest for isosteviol studies. Being a molecule in development, it is important that detailed albumin–isosteviol studies be considered to determine its binding site and kinetics. This will be needed to inform on likely drug-displacement interactions at protein levels that have been reported for similar anionic drugs like salicylates, phenylbutazone, and indomethacin.^{12,13} Further, the likely future trial of isosteviol as a therapeutic agent to address cardiac hypertrophy² will probably require its modeled administration over a period of time. This will often require that toxicity arising from drug-displacement interactions, in likely instances of its potential coadministration with other interventions, is adequately considered.

Although the estimated partitioning of isosteviol into plasma compartments appeared consistent and similar in humans and rats ($f_{b,60 \text{ min}}$ of 0.97 [0.01] in rat vs 0.96 [0.01] in humans), $K_{e/p}$ values varied in a time-dependent manner in rats as shown by the larger coefficients of variation reported in Table 2. The in vitro model for $K_{e/p}$ determination offers rapid equilibration of drug with biological matrix as an advantage over the in vivo technique. This equilibration, however, appears to be slower for isosteviol in rat samples. Further, a sharp increase in mean $K_{e/p}$ values (~ 0.047 vs 0.072) was observed at 30 μM isosteviol concentration in rat blood. This is believed to have resulted from the predominating influence of other interactions at high isosteviol concentration in rat blood where the erythrocyte surface charge is higher¹⁴ but plasma volume and serum albumin^{15,16} content are less compared with human blood. This interspecies differences in blood-component characteristics may also explain the varied equilibration pattern of isosteviol in human and rat blood. In addition, the authors hypothesize that the saturation of protein binding sites at high concentration in rat blood may have also favoured an increased influx of isosteviol into erythrocytes, leading to the observed increase in $K_{e/p}$.

A study of the disposition of isosteviol after single oral and intravenous doses by Jin and et al⁶ reported a mean volume of distribution of 68 mL in rats with average body weight of 319 g. Assuming a multiple frequency bioelectrical impedance analysis value of 0.969¹⁷ and an intracellular water fraction of 0.6,¹⁸ the volume of extracellular water would be approximately 123 mL in the animals studied. The volume of distribution of isosteviol from plasma studies reported by Jin et al⁶ would, hence, amount to about 56% of extracellular water in rats. Although a reference value for isoste-

viol's steady-state volume of distribution in rats is unavailable, it is most likely that such values will be significantly lower than extracellular water volume in rats, reflecting the extensive partitioning of isosteviol into plasma compartments predicted in the present study.

At the moment, the precise biological target of isosteviol is unknown. Hence, the extent to which its partitioning into plasma compartments might affect its efficacy would most likely be influenced by the location of such targets. The profile of isosteviol in blood compartments presented in this study would likely be most critical if receptors at intracellular sites are needed for therapy. This is because only free molecules would be able to access such sites. It is noteworthy that the contributions of other biological factors such as active transport and dissociation rate of protein-bound molecules might also be very important for the overall efficacy of isosteviol.

Conclusions

The present study identified a preferential partitioning of isosteviol into plasma compartments of the blood in humans and rats. Although the potential for isosteviol-induced hematotoxic events looks unlikely, drug interactions at blood protein binding sites might be very important for its safe coadministration with other agents.

CRediT authorship contribution statement

Ayorinde Adehin: Data curation, Formal analysis, Writing - review & editing, Validation. **Keai Sinn Tan:** Data curation, Formal analysis, Writing - review & editing, Validation. **Wen Tan:** Data curation, Formal analysis, Writing - review & editing, Validation.

Conflicts of Interest

The authors have indicated that they have no conflicts of interest regarding the content of this article.

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