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Determination of the polyfructosan sinistrin in biological fluids by HPLC with electrochemical detection

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Abstract

A sensitive HPLC method with electrochemical detection was developed for the determination of the polyfructosan sinistrin in human plasma and urine. Proteins and interfering components such as glucose were removed from plasma and urine samples by solid phase extraction on C_{18} cartridges. Chromatographic separations were achieved at 85°C on a 300 mm × 7.8 mm i.d. column, using ion moderated partition chromatography with distilled water at a flow rate of 0.6 ml min^{-1}. After post-column addition of NaOH 0.3 M (0.6 ml min^{-1}), the electrochemical detection of the eluate was performed with a sequence of three potentials (0.05 V, −0.8 V, 0.6 V) of specific pulse duration 300, 100 and 100 ms respectively. Xylose was used as internal standard for the quantitative determinations. The calibration curves were linear ($r^2 > 0.992$) over the working range 5–300 $\mu g\ ml^{-1}$. This method has been characterized, validated and applied successfully in a study comparing two modes of glomerular filtration rate determination in healthy volunteers (bolus vs. constant rate infusion of sinistrin).

Keywords: Polyfructosan; Sinistrin; Electrochemical detection; Ion-moderated partition chromatography; Glomerular filtration rate; Clearance; Renal function

1. Introduction

Sinistrin (Fig. 1) is a polyfructosan extracted from the red squill (*Urginea maritima* L., Baker, Liliaceae) [1]. This natural product is a reducing carbohydrate polymer, which consists of fructose chains linked to glucose [2]. The exact arrangement of carbohydrate branches has not been completely determined [3].

After parenteral administration, sinistrin is eliminated by the kidney, where it is filtered through the glomerulus with no subsequent reabsorption or secretion by the renal tubular cells. Therefore, sinistrin clearance can be used as a marker of the glomerular filtration rate (GFR) [4].

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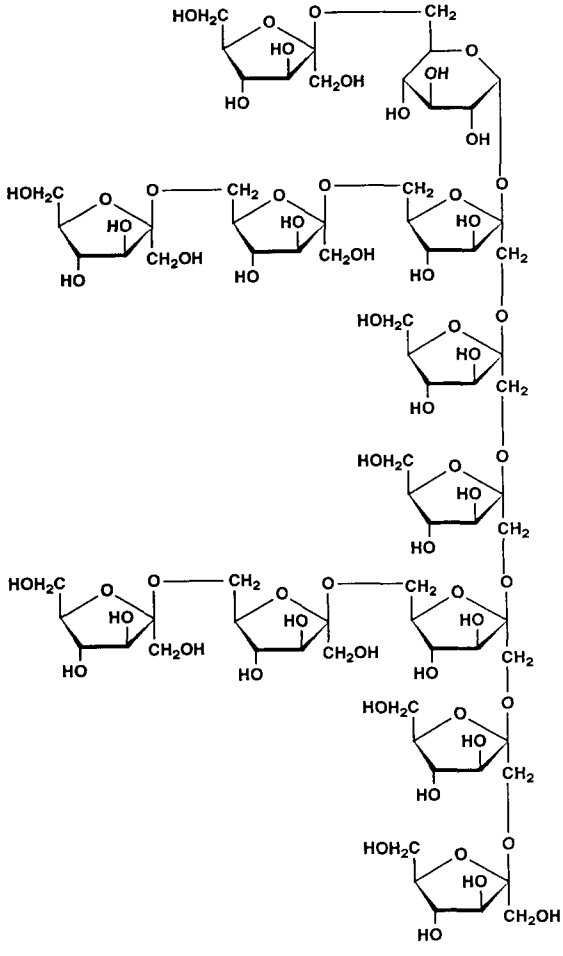

Fig. 1. Proposed chemical structure of sinistrin.

Sinistrin has the advantage over the related polyfructosan inulin of being very water soluble at room temperature. Thus, smaller volumes can be infused at high concentrations (250 mg ml^{-1}) as limpid solution, which may represent a substantial improvement, particularly for children [5].

GFR assessment is traditionally based on the measurement of the renal clearance of inulin calculated from its urinary excretion rate at steady state plasma concentration. This is reached by the administration of an intravenous loading dose followed by a constant rate infusion. This method is not always convenient, is time consuming, needs accurate urine collection and is subject to error due to incomplete bladder emptying. Moreover, classical colorimetric methods (after hydrolysis of inulin to fructose and glucose) have been impeded by the lack of analytical sensitivity and precision and the difficult problem of crossreaction of glucose with the reducing agent, making correction for blanks almost impossible, especially at low concentrations of the marker [6,7]. To overcome the latter analytical limitations, an HPLC method has been proposed to quantify inulin in biological fluids [8].

A sensitive method was developed and validated for the accurate and precise determination of sinistrin in biological fluids (down to 5 μg ml^{-1}), so that its clearance can be calculated not only at the steady state according to the classic urinary clearance method but also from the decrease in plasma concentration following a bolus injection. This was achieved using HPLC combined with electrochemical detection after solid phase extraction (SPE).

The detailed assay validation reported here was performed according to the recommendations of the Conference Report on Bioanalytical Method Validation [9] and was successfully applied in a study comparing two modes of administration of sinistrin in healthy volunteers [10].

2. Experimental

2.1. Chemicals

Sinistrin was obtained as a saline formulation for intravenous administration (Inutest, Laevosan-Gesellschaft m.b.H., Linz/Donau, Austria). Internal standard (IS) xylose and sodium hydroxide were quality puriss. p.a. and supplied by Fluka (Buchs, Switzerland). Methanol (HPLC grade) was purchased from Romil Chemicals (Loughborough, UK). All other chemicals were of analytical grade and used as received. Doubly-distilled water was used after filtration through 0.22 μm pore size GS filters (Millipore, Bedford, MA).

2.2. Standard solutions

Six standard solutions containing sinistrin at 5, 25, 75, 150, 225 and 300 μg ml^{-1} were prepared

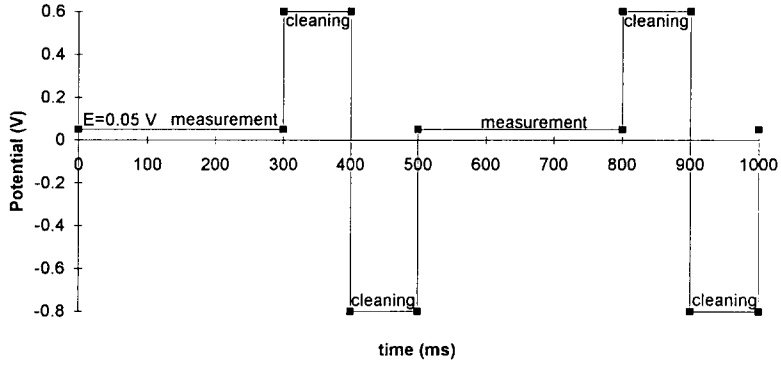

Fig. 2. Successive potentials applied for detection of polyfructosan sinistrin

in pooled blank plasmas and blank urine. Plasma and urine control samples at low (20 μg ml^{-1}), medium (100 μg ml^{-1}) and high (250 μg ml^{-1}) concentrations were similarly prepared. All samples were stored as 650 μl aliquots in polypropylene Eppendorf tubes at −24°C and thawed individually on the day of analysis. Xylose was dissolved in distilled water to make a 100 μg ml^{-1} IS solution. Standard solutions were used to establish the calibration curve, one set at the beginning and a second set at the end of the HPLC run (validation).

2.3. Chromatographic system

The HPLC system used consisted of an AS-2000 A autosampler, a L-6200 A Intelligent pump, a D-2500 Chromato-Integrator (Merck-Hitachi Ltd, Tokyo, Japan) and an HP 1049 A Programmable Electrochemical Detector (Hewlett-Packard, Avondale, PA) with a gold working electrode and an Ag/AgCl reference electrode with potassium chloride as internal electrolyte.

Chromatographic separations were performed on a 30 mm × 4.6 mm i.d. CarboC precolumn connected to a 300 mm × 7.8 mm i.d. Aminex HPX-87C column (Bio-Rad Labs, Richmond, VA) which was kept at 85°C in a column oven (Bio-Rad Column Heater).

The mobile phase was distilled water at a flow rate of 0.6 ml min^{-1}. Post-column addition of 0.3 M sodium hydroxide at a flow rate of 0.6 ml min^{-1} was performed with an SSI 222D pump (Scientific Systems, Inc., PA) connected to a mixing tee (Supelco, Inc., Bellefonte, PA).

2.4. Electrochemical detection

Since single potential oxidation of sugars causes the electrode surface to become contaminated by the products of the oxidoreduction [11], serial sequences of three potentials ($E_1 = 0.05$ V, $E_2 = -0.8$ V, $E_3 = 0.6$ V) had to be applied with specific pulse durations ($t_1 = 300$ ms, $t_2 = 100$ ms, $t_3 = 100$ ms) (Fig. 2). The response time was set at 8.0 s. With these parameters, a stable baseline and an optimal detection of sinistrin and xylose were obtained. A regular mechanical polishing of the gold working electrode is necessary to ensure a clean electrode surface. The cleaning needs to be done only before starting measurements of a series of samples (particularly when the electrode has not been used for some days).

2.5. Sample preparation

Plasma and urine samples were subjected to SPE using Sep-Pak® Plus C_{18} (Waters) cartridges on a Visiprep® Vacuum 12-manifold (Supelco) for the removal of proteins and interfering components like glucose.

The cartridges were conditioned with 2 ml of methanol followed by 3 × 3 ml of distilled water. Samples (0.5 ml, diluted urine (1:1 to 1:100) or plasma) were subsequently applied in duplicate to the cartridges. Interfering material was removed by washing with 5 × 3 ml of distilled water. Sin-

istrin was eluted with 2 × 1 ml of distilled water–methanol (50:50, v/v). The eluted solutions to which 50 μl IS solution was added were evaporated to dryness on a water bath at 37°C under a nitrogen stream. The residues were dissolved in 0.5 ml of water and transferred to microvials from which 20 μl was injected into the HPLC column.

2.6. Validation of the method

Throughout the analysis of biological samples, control samples at three concentration levels (20, 100, 250 μg ml^{-1}) were assayed every five samples. Three injections were performed for each individual sample. The complete validation of this assay was carried out according to the guidelines put forward by the Conference Report on Bioanalytical Methods Validation.

The control samples were used for the determination of the precision and accuracy of the method, precision being calculated as the RSD (%) within a single run (intra-assay) and between different assays (inter-assay), and accuracy as the percentage of deviation between nominal and measured concentrations with the established calibration curves.

The recoveries from plasma and from aqueous solutions were determined as the peak-area response of processed samples expressed as a percentage of the response of pure sinistrin solutions not subjected to SPE [12].

The stability of sinistrin in plasma and in urine was determined as follows:

(a) by storing plasma and urine samples containing sinistrin (250 μg ml^{-1} and 100 μg ml^{-1} respectively) at room temperature for 24 h.
(b) by subjecting aliquots of plasma and urine at various (low, medium, high) concentrations of sinistrin to three freeze–thaw cycles: frozen duplicate samples were allowed to thaw at ambient temperature for 2 h and were subsequently refrozen. Their sinistrin concentration was compared with aliquots that had not been subjected to the freeze–thaw cycles.
(c) The potential influence of the evaporating step (water bath at 37°C) was also tested: samples were left for 6 h at 37°C and at a higher temperature (60°C). Aliquots were injected into the HPLC column at selected time intervals.

The selectivity of the method was determined by spiking sinistrin samples with drugs commonly used in therapeutics (cf. below).

The possible hydrolysis of sinistrin during the course of evaporation, storage and analysis was also assessed. For this purpose, sinistrin in acidic medium (HCl 0.01 M) was incubated in a water bath at 85°C. Aliquots were injected into the HPLC column at selected time intervals. The identity of the peaks of glucose and fructose present on the chromatogram of the incubated material was confirmed by co-injection with authentic samples.

2.7. Pharmacokinetics

The present method was applied to evaluate the renal function of healthy volunteers by bolus injection and by the constant rate infusion techniques. Blood and urine samples were obtained from healthy volunteers according to a research protocol approved by the Ethics Committee of the Institution.

Blood samples were drawn into 2.7 ml EDTA Monovettes and immediately centrifuged at 900*g* for 10 min at 4°C.

In the bolus injection mode, two healthy volunteers (males of 83.2 and 85.5 kg) received 3100 mg of sinistrin (Inutest) over 10 min. Blood samples were taken from the opposite arm at specified time intervals after the start of the infusion.

In the traditional, constant rate infusion approach, the same volunteers received, 1 week later a bolus dose of 3100 mg followed by a constant rate infusion of 21 mg min^{-1} sinistrin. Following a 1 h equilibration period, blood and urine sampling was regularly performed for 5 h.

3. Results and discussion

3.1. Setting-up and validation

Sinistrin and xylose (IS) were well resolved from endogenous plasma and urine components as shown in the typical HPLC profiles (Fig. 3a–c).

The clean-up procedure by SPE was found to be a reliable method for eliminating interfering material from both plasma and urine matrices. The efficiency of the solid phase extraction has been determined with spiked plasma samples or aqueous solutions of sinistrin to assess the influence of the matrix (Table 1). The overall recovery was $103\% \pm 8$ in plasma and $96\% \pm 1$ in water. These results indicate that the matrix or the concentration range of sinistrin have no significant effect on the efficiency of the extraction.

Calibration curves were obtained by unweighted least-squares linear regression analysis of the peak-area ratio of sinistrin to xylose (IS) vs. the concentration of sinistrin in each calibration sample. Typical standard curves were described by $y = 0.014x + 0.1$, in which y is the peak area ratio of sinistrin to IS, and x is the concentration of sinistrin. The regression coefficients were $0.992 < r^2 < 1.000$.

At concentrations higher than 350 μg ml^{-1}, the relation was non-linear due to the saturation at the electrode surface. The high standard concentration of the calibration curve was set as a precaution to 300 μg ml^{-1}. The plasma samples collected immediately after the bolus injection therefore had to be diluted. As the concentration of sinistrin in urine was generally higher than those encountered in plasma, an appropriate dilution (1:100 to 1:1) was applied to urine when necessary.

The precision and accuracy determined during the validation procedure are given in Tables 2 and 3. At low, medium and high concentrations in plasma, the overall intra-assay precision was 8.3%, 0.9% and 3.5% respectively. At the same dose level, the precision in urine was 8.2%, 5.9% and 4.7% respectively. Both plasma and urine quality control samples were also analyzed on different days to assess inter-assay variation.

The limit of quantitation (LOQ) [9] was 5 μg ml^{-1} and was chosen as the lowest concentration of the standard calibration curve. The limit of detection (LOD) was 0.8 μg ml^{-1}. A lower LOQ can be expected by loading a larger sample size on the cartridges, by reconstituting the residues of evaporation in smaller volumes (since sinistrin is very soluble even at high concentration) or by injecting a larger volume of sample into the HPLC column.

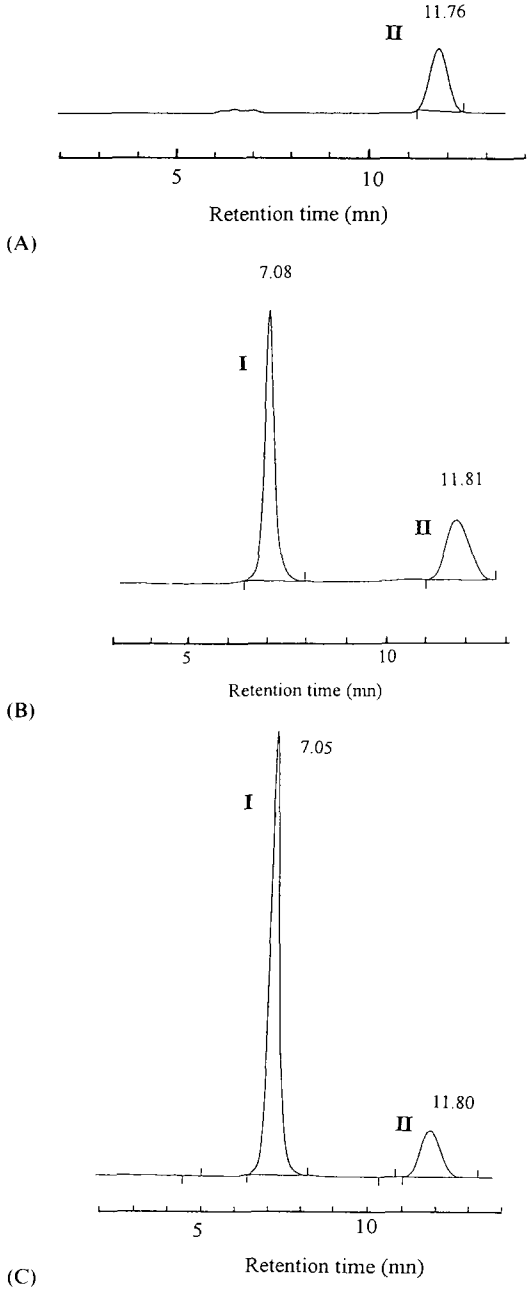

Fig. 3. Chromatograms of (A) blank plasma with IS (xylose (II), 100 μg ml^{-1}, 50 μl), (B) plasma sample and (C) urine sample of healthy volunteers having received sinistrin (I) i.v. to which IS was added.

Table 1
Recovery of sinistrin from plasma and water (matrix effect)

Nominal concentration (μg ml^{-1})	Recovery[a] (%)	
	From plasma sample (mean ± SD)	From aqueous sample (mean ± SD)
20.0	110 ± 7	96 ± 9
100.0	104 ± 4	97 ± 3
300.0	95 ± 3	95 ± 2

[a] Each value represents a mean of $n = 6$ duplicates.

The stability of sinistrin in biological matrix and at various steps of the analysis was explored to assess the substance integrity throughout the procedure, starting from sampling at the bedside to processing in the laboratory. Spiked urine samples (20, 100 and 250 μg ml^{-1}) subjected to three freeze–thaw cycles showed no significant change from the starting concentration (< 0.2% overall). This is in contrast with urine containing inulin, in which precipitation occurs in the samples after thawing. When subjected to the above procedure, plasma samples at 20, 100 and 300 μg ml^{-1} showed consistently an apparent increase (+ 21%, + 11% and + 9% respectively) in the sinistrin concentration. There is no valid explanation for this observation, even though the alteration of the biological matrix after the freezing–thawing process may give rise to decomposition products interfering with the detection of sinistrin during the HPLC analysis. Therefore plasma samples should be immediately centrifuged and frozen, stored at − 20°C and thawed just prior to the analysis.

Table 2
Precision and accuracy of the assay for sinistrin in plasma

Nominal conc. (μg ml^{-1})	Conc. found (μg ml^{-1})	Precision (RSD; %)	Accuracy[a] (Deviation; %)
Intra-assay ($n = 6$)			
20.0	19.7 ± 1.6	8.3	−1.6
100.0	111 ± 1	0.9	11.0
250.0	241 ± 8.4	3.5	−3.8
Inter-assay ($n = 6$)			
20.0	19.6 ± 1.9	9.7	−1.9
100.0	106 ± 8	7.5	6.1
250.0	240 ± 15	6.3	−3.9

[a] $\frac{\text{Found} - \text{Nominal}}{\text{Nominal}} \times 100$

Table 3
Precision and accuracy of the assay for sinistrin in urine

Nominal conc. (μg ml^{-1})	Conc. found (μgml^{-1})	Precision (RSD; %)	Accuracy[a] (Deviation; %)
Intra-assay ($n = 6$)			
20.0	20.7 ± 1.7	8.2	3.6
100.0	97 ± 5.7	5.9	−2.9
250.0	271 ± 12	4.7	8.7
Inter-assay ($n = 5$)			
20.0	20.2 ± 1.9	10.2	1.0
100.0	111 ± 8	8.8	11.1
250.0	237 ± 10	4.2	−5.1

[a] $\frac{\text{Found} - \text{Nominal}}{\text{Nominal}} \times 100$

Plasma samples (250 μg ml^{-1}, $n = 3$) and urine samples (100 μg ml^{-1}, $n = 3$) spiked with sinistrin and allowed to stand at room temperature for 24 h showed a substantial decrease (− 6.5% in plasma and − 9.2% in urine) in the nominal starting concentration. Therefore plasma and urine samples should be immediately processed and stored at − 20°C.

Interestingly, both urine and plasma samples showed no sign of sinistrin precipitation after the freeze–thaw procedure, in contrast to the related polyfructosan inulin. We found no evidence of sinistrin decomposition either in urine or in plasma during storage, since the concentration values of the control samples were stable over time, and the slope of the calibration curve remained constant.

In acidic conditions sinistrin is partially hydrolyzed into its monomeric subunits glucose and fructose. Sinistrin was not hydrolyzed during the evaporation process, as attested by the absence of glucose and fructose peaks on the chromatogram of pure sinistrin subjected to this procedure.

The selectivity of the assay was determined using the following drugs or diagnostic markers: acetylsalicylic acid, 300 mg l^{-1}; ascorbic acid, 15 mg l^{-1}; *p*-aminohippuric acid, 100 mg l^{-1}; chloramphenicol, 150 mg l^{-1}; diazepam, 0.3 mg l^{-1}; lidocain, 5 mg l^{-1}; nitrazepam, 0.3 mg l^{-1}; phenobarbital, 20 mg l^{-1}; quinidin, 4 mg l^{-1}; theophyllin, 20 mg l^{-1}. None of the above (at therapeutic levels) interfered with the analysis.

3.2. Pharmacokinetics

The concentration–time curves of sinistrin in two volunteers following a bolus intravenous injection and a constant rate infusion are shown in Figs. 4 and 5.

The renal clearances (CLs) of sinistrin determined by the kinetic approach based on the plasma disappearance of sinistrin following both the bolus injection and the constant rate infusion or by the classic urinary clearance method are compared in Table 4. The pharmacokinetic parameters were determined according to both a two-compartment model (non-linear regression, SIPHAR program, SIMED Créteil, France) and to a non-compartmental approach. GFRs estimated as the systemic clearance of sinistrin, i.e. without urine collection, either from the bolus injection or from the constant infusion technique, were superimposable in the same patient, even when measured 1 week apart (104 ml min^{-1} vs. 106 ml min^{-1}; 103 ml min^{-1} vs. 110 ml min^{-1}).

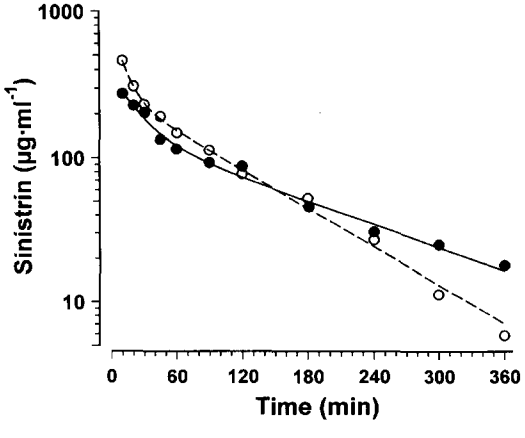

Fig. 4. Plasma concentration–time curves of sinistrin in two healthy volunteers following a bolus infusion (3100 mg over 10 min).

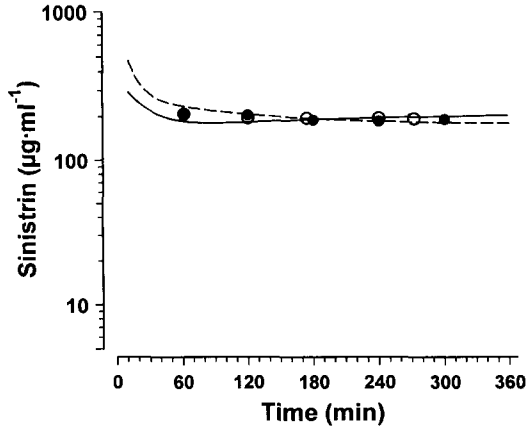

Fig. 5. Plasma concentration–time curves of sinistrin in two healthy volunteers after a bolus (3100 mg) followed by a constant rate infusion (21 mg min^{-1}).

When the GFR was assessed from the urinary excretion rate of sinistrin, the integrated approach (A_e/AUC) gave similar results to the systemic clearance (108 ml min^{-1} and 111 ml min^{-1}). When derived from the various collection fractions obtained by spontaneous voiding, a

Table 4
Comparison of methods for calculating sinistrin renal clearance (ml min^{-1}) after bolus infusion and constant rate infusion

Method	Volunteer 1	Volunteer 2
Bolus infusion		
I. Two compartments model: non linear regression	104	103
II. Non-compartmental model:		
(a) CL = D/AUC	105	102
(b) CL = A_e/AUC	108	111
Constant infusion		
(a) CL = R_{inf}/C_{ss}	106	110
(b) CL = U^*V/P (range)	142 (108–187)	138 (127–150)

CL: clearance (ml min^{-1}); D: dose (mg); A_e: amount excreted (mg); R_{inf}: rate of infusion (mg min^{-1}); C_{ss}: steady state plasma concentration (mg ml^{-1}); U: urine concentration (mg ml^{-1}); P: Plasma concentration (mg ml^{-1}); V: urine flow (ml min^{-1}); AUC: area under the plasma concentration time curve (mg min ml^{-1}).

large random variability was observed in the clearance values. Hydration, and accordingly, urinary flow (V), incomplete bladder emptying, posture, exercise, meals, time delay between filtration at the glomerulus site and the bladder, are all variables known to influence the clearance measured in short time urine collection [13]. The latter gives higher values for sinistrin clearance. The cumulative error of the various measured variables (U, P and V) introduced in the calculation ($U \cdot V/P$) may contribute to the overall variability of this latter approach. The cumulative amount excreted in urine (A_e) is in close agreement with the total dose injected or infused to the volunteers, with a mean recovery of 104%.

4. Conclusion

A reliable quantitative determination of the polyfructosan derivative sinistrin in plasma and urine has been developed and validated using HPLC combined with electrochemical detection. This method represents a substantial improvement in the sensitivity for the quantitative analysis of the polyfructosan sinistrin (down to ≈ 5 μg ml^{-1}), and improves the accuracy of GFR determination, enabling its clearance to be determined both after bolus injection and after a constant rate infusion of low dose, with or without simultaneous urine collection.

This method represents a useful tool in studies on renal pathophysiology and for the assessment of the influence of drugs on the kidney. The possibility to assess GFR as sinistrin clearance without urine collection may greatly improve the utility and the acceptability of this approach in clinical settings.

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