Journal of Chromatography B, 885–886 (2012) 24–29

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

A validated high-performance liquid chromatographic method with diode-array detection for the estimation of xyloketal B in rat plasma

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ARTICLE INFO

Article history:
Received 30 July 2011
Accepted 4 December 2011
Available online 13 December 2011

Keywords:
Xyloketal B
Pharmacokinetics
HPLC-DAD
Rat plasma

ABSTRACT

A sensitive and specific HPLC–UV method was developed and validated for the determination of xyloketal B in rat plasma. Following liquid–liquid extraction, the separation was performed using an isocratic mobile phase of methanol–acetonitrile–water (30/30/40, v/v/v) on a Phenomenex C_{18} column (4.6 mm × 250 mm, 5 μm). The eluent was monitored at 220 nm and at a flow rate of 0.8 ml min^{-1}. A linear curve over the concentration range of 1–128 μg/ml ($r > 0.999$) was established. The LLOQ of the method was 1 μg/ml. Good precision and accuracy at concentrations of 2.5, 25 and 100 μg/ml were obtained. The recovery of xyloketal B in plasma was >87.91%. The validated method was found to be specific, precise and accurate in the study. The analytic method was satisfactorily applied to perform preclinical pharmacokinetic study of xyloketal B in rat plasma.

1. Introduction

Atherosclerosis, a long-term chronic disease characterized by a bland lipid storage and fibrous connective tissue in the large arteries, is the main cause of cardiovascular disease [1,2]. Despite continuous research efforts, cardiovascular disease continues to be the major cause of mortality and morbidity in many countries [3–6].

Marine microorganisms can yield various of novel metabolites owing to their characteristic growing environment [7–10]. Xyloketals are a series of novel products isolated from marine mangrove endophytic fungus from the South China Sea [11,12]. One of the derivatives from the fungus *xylaria* sp. (no. 2508) [11], xyloketal B, is a novel benzopyran compound with unique structure (Fig. 1). In our previous study, xyloketal B exhibited multiple and strong pharmacological properties, such as blood vessels relaxing effect, and the effect of inhibiting the L-calcium channel of hippocampal cells [13,14]. In addition, xyloketal B significantly protected human umbilical vein endothelial cell (HUVEC) from injury from oxidatively modified low density lipoprotein (oxLDL) without either toxic or proliferative effects in vitro [15]. The remarkable anti-atherosclerosis effect may serve xyloketal B as a promising treatment of this disease. Furthermore, xyloketal B was found to protect PC12 cells against oxygen glucose deprivation (OGD) – induced cell injury [16]. Based on the strong pharmacodynamic activities of xyloketal B, it is necessary to characterize the pharmacokinetic property of this interesting compound.

The discovery and development of new drug candidates is a long-term and fund-consuming business [17]. Statistical analysis has shown that, to a great extent, the termination of the development of a considerable number of drug candidates owes to their poor pharmacokinetic characteristic [18–21]. Therefore, pharmacokinetics data play a crucial role in drug development and further consideration.

In view of the above, the decision to proceed with new drug candidates beyond the preclinical stage is a critical step. The aim of this study is to develop and validate a simple, reproductive and selective HPLC method for the determination of xyloketal B in rat plasma. To our knowledge, this is the first report on the pharmacokinetics of xyloketal B.

2. Materials and methods

2.1. Chemicals and reagents

Xyloketal B (99%) and compound 1(internal standard 97.4%) (Fig. 1) was synthesized by Sun Yat-Sen University (Guangzhou, Guangdong, China). HPLC-grade reagents such as acetonitrile and

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doi:10.1016/j.jchromb.2011.12.005

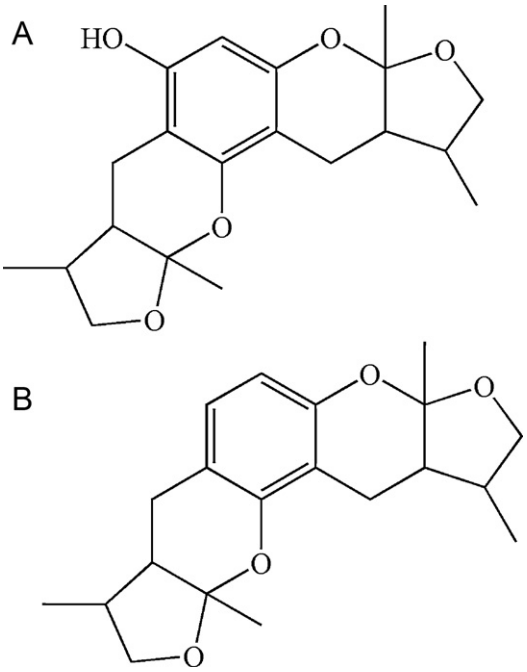

Fig. 1. Chemical structure of (A) xyloketal B and (B) compound 1 (internal standard).

methanol were purchased from Merck (Darmstad, Germany). All other analytical grade reagents such as ethyl acetate and phosphoric acid were obtained from commercial sources. Ultra-pure water was prepared in the laboratory using a Sartorius Arium611VF system.

2.2. Chromatographic instruments and conditions

HPLC separations were performed on a Shimadzu LC-2010C system (Kyoto, Japan) with a quaternary pump, an online degasser, an automatic sample injector, a temperature controlled column compartment, a photodiode array (PDA) detector (SPD-M10AVP), and a data system (Shimadzu' LC solution). Chromatographic separation was carried out on a Phenomenex C_{18} column (4.6 mm × 250 mm, 5 μm) associated with a C_{18} precolumn (Phenomenex). The mobile phase consisted of methanol–acetonitrile–water (30:30:40, v/v/v) using an isocratic elution. The flow rate was 0.8 ml min^{-1}. All solutions were filtered under vacuum through a 0.45 μm membrane filter and degassed ultrasonically before their using. Compound 1 was used as an internal standard (IS). The column temperature was maintained at 35 °C. Detection was performed at 220 nm and the injection volume was 20 μl.

2.3. Preparation of the calibration standards and quality control (QC) samples

Stock solutions (1.0 mg/ml) of xyloketal B and compound 1(IS) in methanol were prepared and stored at 4 °C. The working stock solutions (25 μg/ml and 100 μg/ml) of xyloketal B were prepared from the primary stock solution by a mixture of methanol–acetonitrile–water, 30:30:40, v/v/v (diluent). The calibration standard curve was in the range of 1–128 μg/ml. It was constructed by spinking untreated plasma with different working standard solutions and 10 μl of stock solutions of IS (50 μg/ml). Low, medium, and high concentration quality control (QC) samples at different levels of 2.5, 25 and 100 μg/ml were prepared in the same manner. One calibration curve was constructed on each analysis batch using freshly prepared calibration standards, and the quality controls should be incorporated in each assay run.

2.4. Sample preparation

Xyloketal B was extracted from the plasma using a liquid-liquid extraction. All frozen rat plasma samples were thawed at the room temperature before processing. Then, 10 μl of IS 1 mg/ml was added to 500 μl of blank plasma. After mixing, 500 μl of 0.1 M phosphoric acid was added. Following the vortexing of 1 min, 5 ml of ethyl acetate was added into the tube, the mixture was vortexed for 2 min and centrifuged at 2500 × *g* for 10 min. After that, the supernatant was carefully transferred to a glass tube and the extraction step was repeated twice. The extracts were combined and evaporated to dryness in a water bath at 45 °C under a gentle stream of nitrogen. Before analysis, the residue was reconstituted in 200 μl of mobile phase and vortexed for 1 min. After centrifugation at 12,000 × *g* for 15 min, a 20 μl of the supernatant was injected into the HPLC system.

2.5. Validation of the method

The developed method was validated according to the Food and Drug Administration (FDA) guidelines for analysis of biological samples [22].

2.5.1. Specificity

The specificity of the method was tested by analyzing six different drug-free plasma samples from six rats. Each blank sample was handled by the procedure described in Section 2.4 and confirmed that endogenous substances did not interfere with the analyte and the internal standard.

2.5.2. Linearity

The linearity of the method was evaluated by a calibration curve in the concentration range of 1–128 μg/ml. The calibration curve was constructed by plotting the peak area ratio of analyte to IS versus analyte concentration with a weight of $1/x^2$.

2.5.3. Limit of quantification and detection

The limit of quantification (LOQ) was defined as the lowest concentration on the standard calibration curve. It was established by determining the concentration of at least five spiked calibration standards and the signal-to-noise ratio of 10:1 was used. Also, the precision and accuracy were assessed at the lowest concentration of the standard calibration curve.

The limit of detection (LOD) was defined as the lowest concentration of drug in spiked plasma samples based on a signal-to-noise ratio of 3:1.

2.5.4. Assay precision and accuracy

The precision and accuracy of the method were assessed by assaying QC samples at three different concentrations (2.5, 25 and 100 μg/ml). Five replicate of each level were analyzed in 1 day or over three consecutive days. Accuracy was expressed as the percentage of observed value to true value, and precision was expressed as the percentage relative standard deviations (RSD, %).

2.5.5. Extraction recovery

The recovery showed an ability to extract the analyte from the test biological samples. Recovery of xyloketal B by ethyl acetate extraction was determined at three different levels (2.5, 25 and 100 μg/ml) ($n = 5$). The recovery of xyloketal B was calculated by comparing the analyte observed peak area of spiked QC samples in five replicates with those from the non-processed standard solutions at the same concentration. The recovery of IS was determined similarly.

2.5.6. Stability

To ensure the reliability of the results with regard to handling and storing of the plasma samples and stock standard solutions, stability studies were carried out on QC samples at concentrations of 2.5, 25 and 100 μg/ml. The protocol for the stability assay comprised freeze-thaw stability, short-term and long-term stability, and stock solutions stability. During the freeze-thaw stability assay, the samples were thawed at the ambient temperature without any assistance, and then kept in the freezer (−20 °C) again for minimum of 12 h before carrying out the next thawing, until accomplished three freeze and thaw cycles. The QC samples stored at room temperature for 12 h were evaluated for short-term stability. The long-term stability was determined by analyzing the QC plasma samples after 30 days of storage of −20 °C. Stock solutions stability was tested for 30 days at 4 °C. The resulted stabilities for these samples were then compared with those of the freshly prepared samples.

2.6. Application of the method to a pharmacokinetic study in rats

The developed analytical method was applied to study the plasma concentration–time profile after the intravenous administration of 15, 30 and 60 mg/kg xyloketal B to rats. Male Sprague–Dawley rats (250 ± 10 g) were randomly divided into three groups ($n = 6$) based on the various administration dosages. Animals were fasted overnight with free access to water before the experiment. Animal welfare and experiment procedures were strictly in accordance with the ethical guidelines for investigations in laboratory animals. The blood samples were collected into heparinized tubes at times of 0, 5, 10, 20, 30, 45, 60, 90 and 120 min after intravenous administration. After centrifugation at 3000 × *g* for 15 min, plasma were separated and stored at −20 °C in Eppendorf tubes until HPLC analysis.

2.6.1. Data and statistical analysis

Plasma concentration vs. time profiles were analyzed using the 3P87 software program developed by the Chinese Pharmacological Society to estimate the type of compartment model and pharmacokinetic parameters ($t_{1/2}$, AUC, Cl, etc.). Data were expressed as mean ± S.D.

3. Results and discussion

3.1. Selection of chromatographic conditions

In the present study, several C_{18}-reverse phase columns (Phenomenex, Diamonsil, Dalian Elite) were compared on the basis of the peak shape and retention times. Finally, the Phenomenex C_{18} column was chosen to determine xyloketal B in rat plasma for good peak shape and acceptable retention times (data not shown). The formulation of the mobile phase is another important factor for separating xyloketal B from IS and endogenous components. In order to achieve a suitable mobile phase, several solvent mixtures were tested, including acetonitrile, methanol, and water of various ratios. Under these conditions, the interference of endogenous peaks was the major issue for a good resolution between the analyte and biomatrix. The effective separation in chromatogram was achieved when the mobile phase was composed of 30% methanol, 30% acetonitrile and 40% water.

The maximum absorption of the elution was at 220 nm when detected under pan-wavelength scanning (190–600 nm). Furthermore, it was not sensitive enough to measure the analytes while the UV detector was set at a high wavelength.

3.2. Sample preparation

During analytical method development, direct protein precipitation with popular precipitating agents produced low recovery with high background noise. Furthermore, despite the high performance of the SPE, it was time-consuming and expensive for the preparation of abundant plasma samples. Thus, liquid extraction was selected to carry out the quantitative analysis.

Before extracted by ethyl acetate, plasma samples were acidified with phosphoric acid, which was used to isolate and concentrate the analyte. As a result of this acidified sample preparation, we improved the extraction recovery of xyloketal B and reduced the interference from the endogenous substance.

The sample preparation procedure which consisted of ethyl acetate extraction with the evaporation to dryness following was simple and provided high recovery for both xyloketal B and IS. Other extraction procedures, such as employing dichloromethane as a comparable extracting agent, were studied and showed low recovery and detectable interference from plasma matrix. Therefore, ethyl acetate was chosen as the appropriate extraction solvent. During the IS method, various compounds were tested to decide on a suitable IS which gave satisfactory validation results of RP-HPLC quantification. Among these, compound 1 was selected as the IS because of its stability and significantly high recovery. Furthermore, it performed good separation from biomatrix of the plasma sample and xyloketal B in the assay described.

3.3. Method validation

3.3.1. Specificity

The representative chromatograms of blank rat plasma (A), rat plasma spiked with xyloketal B (16 μg/ml, Fig. 1A) and IS (50 μg/ml, Fig. 1B) (B) and a rat plasma sample 20 min after intravenous injection of xyloketal B (15 mg/kg) are shown in Fig. 2. Xyloketal B and IS peaks were separated completely. No endogenous interfering peaks were observed at the retention times of xyloketal B and IS, thereby confirming the specificity of the analytical method.

3.3.2. Linearity, LLOQ, LOD

The calibration curve of xyloketal B in rat plasma was linear in the concentration range of 1–128 μg/ml (1, 2, 4, 8, 16, 32, 64 and 128 μg/ml). The regression equation of the calibration curve (obtained from eight points) was $y = 0.0412x + 0.0891$ with a correlation coefficient of 0.9998, where y was the peak area ratio of xyloketal B to the IS, and x was the plasma concentration of xyloketal B. LLOQ was tested by determining the concentrations of five spiked calibration standards. The LLOQ of the bioanalytical method was 1 μg/ml for xyloketal B in rat plasma, with a precision of <20% and an accuracy between 80% and 120% for both intra-and inter-day assays. The LOD was estimated to be 0.5 μg/ml based on a signal-to-noise ratio of 3:1.

Results mentioned above showed good linearity, demonstrating that the method could be applied to plasma samples regardless of low or high xyloketal B levels.

3.3.3. Precision and accuracy

The precision and accuracy of the method were determined on three different levels of 2.5, 25, and 100 μg/ml. The results are shown in Table 1. The precision of the assay was expressed by the RSD of the mean value from the nominal concentration; the accuracy was evaluated by the percentage of the mean calculated concentration from the nominal concentration. The intra-day and inter-day precision (RSD %) were within 5.75% for xyloketal B, and the accuracy was higher than 92.80%. These values were within the

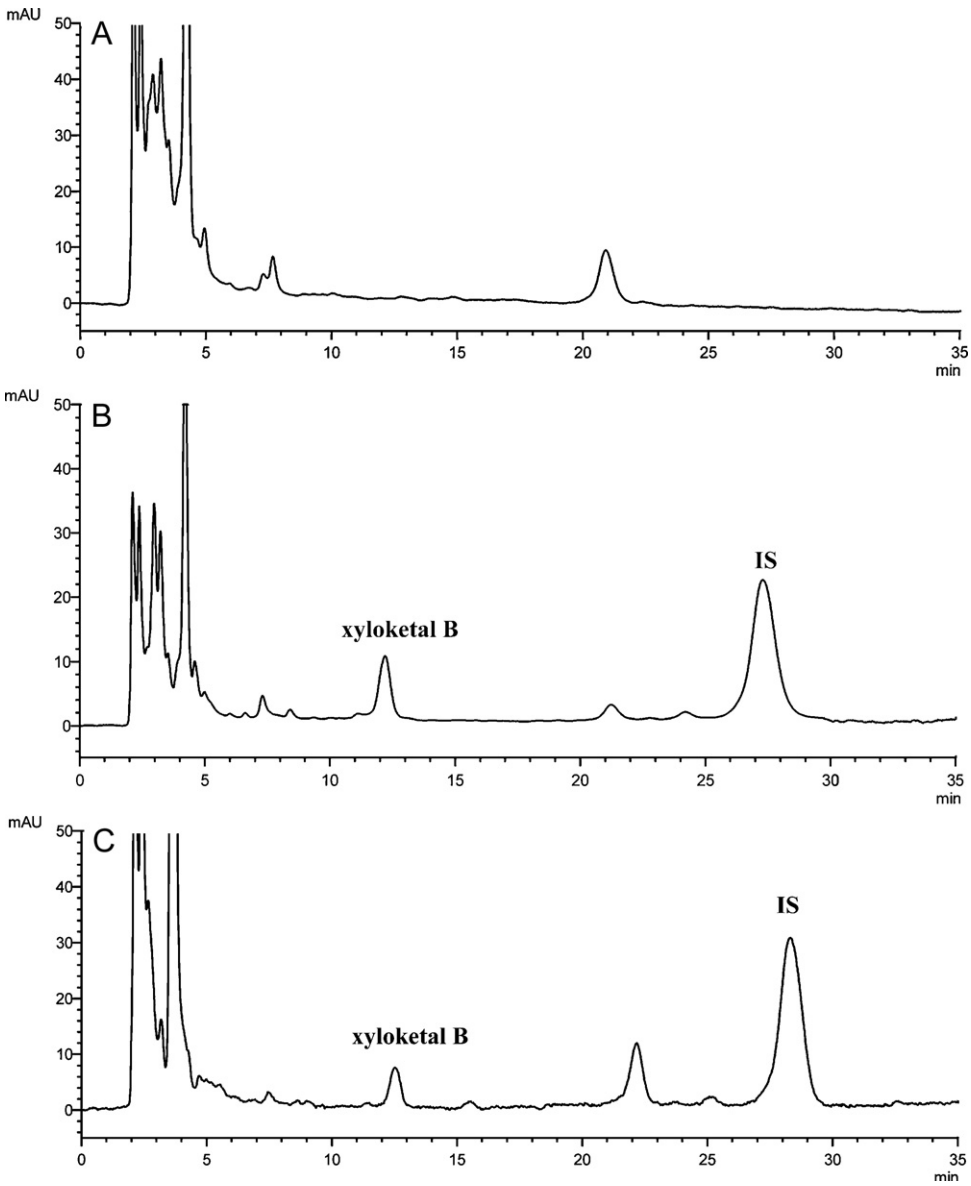

Fig. 2. Representative HPLC chromatograms of (A) blank plasma; (B) blank plasma spiked with xyloketal B (16 μg/ml, Fig. 1A) and IS (50 μg/ml, Fig. 1B); and (C) a rat plasma sample 20 min i.v. administration of xyloketal B (15 mg/kg). Xyloketal B (t_R = 12.3 min); IS (t_R = 27.5 min).

acceptable range, indicating that the developed method was reproducible, accurate, and reliable for the quantitative determination of xyloketal B in rat plasma.

3.3.4. Recovery

The mean (±S.D.) recovery of xyloketal B from plasma sample was 90.80 ± 7.40, 89.85 ± 5.02 and 87.91 ± 2.32 at 2.5, 25, 100 μg/ml, respectively (n = 5). The recovery of IS from rat plasma was 81.8 ± 3.7 at 50 μg/ml.

3.3.5. Stability

Stability tests were performed at the low, medium and high QC samples as described in Section 2.6. The results are summarized in Table 2. Stock solution of xyloketal B and IS was stable for at least 30 days when stored at 4 °C. After 12 h at room temperature, at least 98.05% of xyloketal B was still present in rat plasma. After long-term storage in plasma at −20 °C for 30 days, the concentration of xyloketal B ranged from 96.80% to 98.62% of the initial concentration. The freeze-thaw cycles did not change the concentration of xyloketal B significantly. The average value in QC samples was

Table 1
The validation of intra- and inter-day precision and accuracy with QC samples (n = 5).

Concentration (μg/ml)	Mean calculated concentration(μg/ml)		Precision (RSD %)		Accuracy%	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
2.5	2.46 ± 0.19	2.32 ± 0.10	4.10	5.75	98.40	92.80
25	26.34 ± 1.04	23.78 ± 0.89	2.66	5.25	105.36	95.12
100	96.15 ± 3.61	93.74 ± 4.30	1.42	1.23	96.15	93.74

Table 2
Results for determination of xyloketal B stability ($n = 3$).

Concentration (μg/ml)	Recovery (%)			
	Freeze-thaw stability	Short-term stability	Long-term stability	Stock-solution stability
2.5	95.02	98.05	97.40	102.41
25	95.87	98.46	96.80	99.53
100	97.34	101.57	98.62	103.70

Table 3
Pharmacokinetic parameters of xyloketal B after i.v. administration of 15, 30 and 60 mg/kg ($n = 6$).

Parameters	Unit	15 mg/kg	30 mg/kg	60 mg/kg
V_c	L/kg	0.24 ± 0.02	0.36 ± 0.06	0.13 ± 0.02
$t_{1/2\alpha}$	min	2.17 ± 0.10	2.76 ± 0.35	1.22 ± 0.06
$t_{1/2\beta}$	min	22.49 ± 4.38	26.06 ± 5.56	26.91 ± 4.39
AUC	μg/ml min	575.72 ± 132.2	1275.17 ± 207.6	3575.05 ± 424.7
CLs	L/min	0.04 ± 0.03	0.03 ± 0.02	0.02 ± 0.02

$t_{1/2\alpha}$, distribution half-time; $t_{1/2\beta}$, elimination half-time; Cl, clearance; AUC, the area under the concentration–time curve; V_c, apparent volume of the central compartment; data are expressed as mean ± SD.

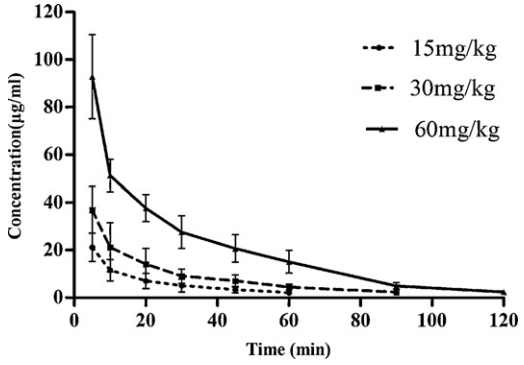

Fig. 3. The Mean concentration–time profiles after i.v. administration of xyloketal B (15, 30 and 60 mg/kg).

95.02% of the initial value after three cycles handling. These results indicated that xyloketal B was stable enough to be analyzed during this assay method.

3.4. Pharmacokinetic (PK) application in rats

The validated HPLC-DAD method was used for determining xyloketal B in rat plasma after intravenous administration at dosage groups of 15, 30 and 60 mg/kg xyloketal B. Fig. 3 illustrates the mean xyloketal B concentrations-time profiles in the plasma. Mean plasma concentrations over time were shown in a dose-dependent manner. A two-compartment model was the best fit for the plasma concentration–time curves obtained in rats. The PK parameter values are listed in Table 3. The half-lives obtained were 22.49, 26.06 and 26.91 min at the dosage of 15, 30 and 60 mg/kg, respectively. In this study, the short half-life suggested that xyloketal B was eliminated in a short time. The plasma concentration–time profiles of xyloketal B in rats demonstrated that it was eliminated rapidly from rat plasma in the first 20 min. A dose proportionality study indicated that there was a good correlation between AUC and dose. Furthermore, xyloketal B showed a linear dynamic characteristic in rats. There was no significant difference in the clearance at three dose levels.

4. Conclusions

A simple, selective and sensitive HPLC-DAD method for the determination of xyloketal B in biosamples was developed and validated. The criteria of precision, accuracy, and recovery for analyzing biosamples were acceptable within the guidance of biological samples analysis. After the intravenous administration of xyloketal B to rats at three different dosages (15, 30 and 60 mg/kg), pharmacokinetic characterization in rats revealed that xyloketal B followed a two-compartment model, including a distribution phase and an elimination phase. The average elimination half-lives were 22.2, 26.06 and 27 min, respectively. The short half-life of xyloketal B examined in this assay indicates that xyloketal B is quickly distributed in the rat and also quickly eliminated. On the other hand, xyloketal B should produce few side effects based on this. The AUC (575.7, 1275.2 and 3575.1 μg/ml min) increased dose-dependently of xyloketal B. The values of $V(c)$ in the three different dosages were 0.24, 0.36 and 0.13 L/kg, respectively. The result exhibits that xyloketal B is mainly distributed in extracellular fluids.

In summary, we have developed a suitable method for the determination of xyloketal B in rat plasma. To our knowledge, this is the first description of xyloketal B pharmacokinetics in the literature. The pharmacokinetic characteristics observed will be useful to further elucidate the mechanism of xyloketal B. The results may also apply to support and enhance the selection and design of the drug candidate for ideal PK properties.

Acknowledgements

This study was supported by National Key New Drug Creation Program (2009ZX09103-039) from the Ministry of Science, Technology of the People's Republic of China, the National Natural Science Foundation of China (20802094), and from Guangdong Provincial Department of Science and Technology, China (2009B090300449).

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