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Journal of Chromatography B, 705 (1998) 105–110

JOURNAL OF CHROMATOGRAPHY B

Sensitive method for the determination of KC 12291 in rat plasma and urine by high-performance liquid chromatography

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Received 17 January 1997; received in revised form 23 September 1997; accepted 25 September 1997

Abstract

A sensitive and selective ion-pair reversed-phase HPLC method has been developed for the quantitative determination of KC 12291 and its major metabolite, KC 13194, in rat plasma and urine. An Ultrasphere ODS column constructed by using a mobile phase of 1 m*M* 1-octanesulfonic acid containing acetonitrile–0.1 *M* triethylamine phosphate buffer, pH 2.2 (29:71, v/v in plasma and 27:73, v/v in urine), an internal standard and a fluorescent detector (excitation 265 nm, emission 370 nm) were used for the separation and the quantitative determination, respectively. The plasma samples were made alkaline and both compounds were cleaned up by the use of liquid–liquid extraction. The limit of quantification was 10 ng/ml for KC 12291 in plasma and urine and for KC 13194 and 100 ng/ml in plasma, respectively. The assay has been validated with respect to system suitability, accuracy, precision, recovery, stability and ruggedness. All validated parameters were found to be within the necessary limits. © 1998 Elsevier Science B.V.

Keywords: KC 12291

1. Introduction

KC 12291, 3-{3-[*N*-[2-(3,4-dimethoxyphenyl)-ethyl)-*N*-methylamino]-propyloxy}-5-phenyl-1,2,4-thiadiazol hydrochloride (Fig. 1), is a cytoprotective cardiovascular agent. As part of the preclinical ADME program and to establish the pharmacokinetics of the drug, it was necessary to determine its concentration and that of the major metabolic breakdown products in plasma and urine. In this report a HPLC method has been developed for the determination of KC 12291 in rat plasma and urine that allows not only the use of an internal standard but also the measurement of its main metabolite, KC 13194 (Fig. 1) (*N*-desmethyl derivative of KC 12291) and of sequences of samples with sufficient stability that have a limit of quantification as low as 10 ng/ml for the parent compound. The method for the determination of KC 12291 was extensively validated in plasma and urine fulfilling international guidelines [1,2]. Method validation included system suitability, linearity, precision, accuracy, specificity, stability, recovery and ruggedness. The validation procedure for measurement of KC 13194 in plasma was limited to the determination of precision, linearity and accuracy.

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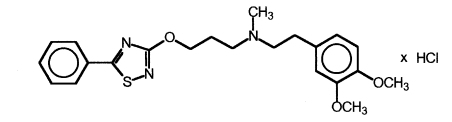
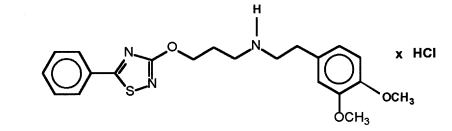
0378-4347/98/$19.00
PII S0378-4347(97)00480-5



CHEMICAL STRUCTURE OF KC 12291



CHEMICAL STRUCTURE OF KC 13194

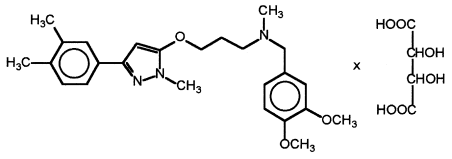


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Fig. 1. Structures of KC 12291, KC 13194 and I.S.

2. Experimental

2.1. Materials and reagents

KC 12291, the internal standard, *N*-(3,4-dimethoxybenzyl)-*N*-{3-[(1-methyl-3-(3, 4-dimethylphenyl)-pyrazol-5-yloxypropyl}methylamine tartarate (I.S.) and KC 13194 (Fig. 1) were obtained from Solvay Pharmaceuticals (Hannover, Germany). Sodium dihydrogen phosphate monohydrate, *ortho*-phosphoric acid (85%) and ammonium hydroxide (25%) were purchased from Reanal (Budapest, Hungary). Solvents were of analytical and HPLC grade. Diethyl ether and triethylamine (TEA) were purchased from Merck (Darmstadt, Germany), methanol (MeOH) was purchased from Carlo Erba (Milan, Italy) and acetonitrile from Interkémia (Budapest, Hungary). Water, purified by the Mill-Q system (Millipore, Bedford, MA, USA), was used in all procedures involving water.

2.2. Apparatus and chromatographic conditions

The HPLC apparatus (Bromma, Sweden) consisted of an LKB a Model 2150 LKB pump, a Model 2152 controller, an RF-551 Shimadzu Fluorescence HPLC Monitor (Kyoto, Japan) (265 nm excitation, 370 nm emission) a Model 2157 LKB autosampler. The cooling device in the autosampler was kept at 5°C. The integration was performed using the Model 2600 chromatography software Rev. 5.00 M3 1988 Nelson system installed on a IBM compatible PC connected to a HP-520 Deskjet printer. The analytical column, an Ultrasphere ODS, 3 μm spherical 80A, 77×4.6 mm I.D., was supplied by Beckmann (Fullerton, CA, USA). The analytical column was kept at room temperature. For analysis of plasma extracts the composition of the mobile phase was 1 m*M* 1-octanesulfonic acid containing AcN–0.1 *M* triethylamine phosphate (TEAP) buffer, pH 2.2 (29:71, v/v). For analysis of urine extracts the composition of mobile phase was 1 m*M* 1-octanesulfonic acid containing AcN–0.1 *M* TEAP buffer, pH 2.2 (27:73, v/v). The flow-rate of the mobile phase was set to 1.0 ml/min. Varian C_{18} cartridges (1-ml volume) Analytichem Bond-Elut® used at room temperature were manufactured by Varian (Harbor City, CA, USA).

2.3. Preparation of stock solution, calibration levels and validation samples

A stock solution of 0.1 mg/ml KC 12291, KC 13194 and an internal standard were prepared in ethanol. The solutions were stable at 4°C for 1 month. Calibration levels of KC 12291 were prepared yielding a concentration range from 10 to 1000 ng/ml, as well as from 10 to 2000 ng/ml in plasma and urine. The range of the calibration levels of KC 13194 in plasma was between 100 and 2500 ng/ml. Validation samples of KC 12291 at three different concentration levels were prepared leading to concentrations of 30, 300 and 900 ng/ml, as well as 60, 800 and 1800 ng/ml in plasma and urine. Validation samples of KC 13194 were prepared in plasma at concentrations of 300 and 900 ng/ml. All calibration levels and validation samples were prepared freshly before the analysis except the samples for stability. The samples of KC 12291 for stability study at two concentration levels 30 and 900 ng/ml, as well as 60 and 1800 ng/ml were prepared in plasma and urine and stored frozen at −20°C for eight weeks. Calibration samples and validation samples were pretreated in exactly the same way as unknown samples.

2.4. Drug administration

KC 12291 was administered to male and female rats. The oral and intravenous doses were 40, 60 and 80 mg/kg body weight, as well as 2, 4 and 6 mg/kg body weight. The rats were fasted overnight. Plasma samples were stored at −20°C until analysis. KC 12291 and KC 13194 were determined in plasma between 0 and 48 h.

2.5. Sample treatments

2.5.1. Plasma

Sodium citrate solution (3.8%) was used as anti-coagulant. The ratio of the blood sodium citrate solution was chosen to be 9:1, v/v. The aliquots of sodium citrate solution were added to the 10-ml tubes and freeze-dried and then the required volumes of the blood were added to the tubes and the tubes were shaken manually. Plasma was separated from whole blood by centrifugation at 3000 rpm for 15 min and was immediately stored at −20°C.

2.5.2. Urine

Rat urine was collected, pooled and stored at −20°C.

2.5.3. Plasma extraction

To 0.5 ml of plasma, 15 ng of internal standard (from 1.5 ng/μl ethanol solution in 10 μl volume) and 0.2 ml of 25% NH_4OH solution were added, then the KC 12291 and its metabolite were extracted with 2 ml of diethyl ether. After centrifugation at 3000 rpm for 15 min, the aqueous layers were frozen in a mixture of acetone–dry ice and the ether layers were transferred into 6-ml glass tubes and evaporated to dryness with nitrogen at room temperature. The evaporation residues were dissolved in 100 μl of MeOH–H_2O (1:1, v/v) and 20-μl aliquots were injected onto the column.

2.5.4. Urine extraction

To 0.5 ml of urine, 15 ng of internal standard (from 1.5 ng/μl ethanol solution in 10 μl) and 0.5 ml of pH 7 0.1 *M* TEAP buffer were added. The sample was homogenized by vortex-mixing for 5 s, then poured onto a BondElut® C_{18} (1 ml) column (SPE) previously activated with 1 ml of MeOH and 1 ml of pH 7 0.1 *M* TEAP buffer. The column was washed with 1 ml of H_2O and 1 ml of AcN–H_2O (20:80, v/v). The compounds under investigation were eluted with 1 ml of MeOH. The methanol eluates were evaporated and the residues were reconstituted in 100 μl of MeOH–H_2O (1:1, v/v) and 30-μl aliquots were injected onto the column.

2.6. Validation of the assay

In a system suitability test, 0.1 ng of KC 12291, 3 ng of KC 13194 and 3 ng of internal standard were injected ($n=6$) from their own working solutions onto the HPLC column. The accuracy and precision of the method and the linearity of the calibration curves were determined intra- ($n=6$) and inter-day (on six successive days) in plasma and urine. The absolute recovery of the parent compound following sample clean up procedure at each calibration point of KC 12291 was assessed from three replicate analyses in plasma and urine. Stability of KC 12291 was tested at −20°C prior to the sample preparation and the freeze–thaw study in three cycles was carried out in plasma and urine. The ruggedness of the method (column to column) was investigated in plasma.

3. Results and discussion

3.1. Reproducibility of the chromatographic parameters

Based on the system suitability test the precision (C.V.) was ≤5% and the accuracy of the chromatography system was between 92 and 108%.

3.2. Specificity, linearity and sensitivity

The specificity of the methods was determined by the chromatographic parameters of KC 12291, 13194 and I.S. (retention times, capacity factors, asymmetry factors, resolution between the test compounds and internal standard) and by screening drug-free pooled rat plasma and urine. KC 12291 and KC 13194 were investigated for interferences with peaks of blank plasma and urine. Representative chromatograms of plasma extract 1.5 h after an oral dosing of 60

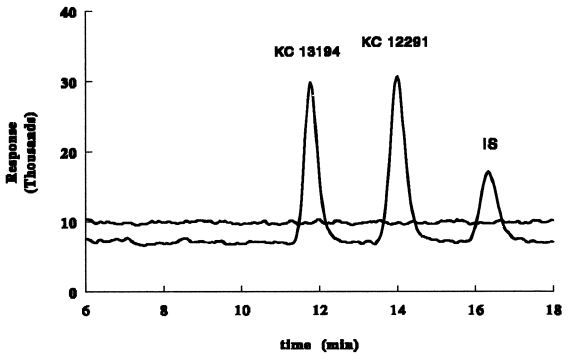

Fig. 2. Chromatograms of a control rat plasma extract and a female rat plasma extract 1.5 h after 60 mg/kg KC 12291 oral dosing. The measured concentration of KC 12291 was 711.0 ng/ml. Conditions: mobile phase: 1 m*M* 1-octanesulfonic acid containing AcN–0.1 *M* TEAP buffer, pH 2.2 (29:71, v/v). Detection: fluorometry, emission: 370 nm, excitation: 265 nm. Flow-rate: 1 ml/min. The analytical column was an Ultrasphere-ODS 80A, 3 μm, 75 mm×4.6 mm I.D..

mg/kg body weight KC 12291 (Fig. 2) showed that the KC 12291 and KC 13194 were completely resolved from each other, from the internal standard and from any endogeneous peaks in plasma. In the blank urine extract, no interferences were found between the endogenous peaks and KC 12291 and I.S. After the extraction described above for the evaluation of the calibration curves linear least squares regression of KC 12291/I.S. and KC 13194/I.S. peak area ratios vs. standard KC 12291 and KC 13194 concentrations were performed. The intra- (y=3.988E−03x−0.0101, r^2=0.9998) and inter-day (y=4.010E−03x−0.0022, r^2=0.9998) calibration curves were linear in the 10–1000 ng/ml plasma concentration range for KC 12291. The intra- and inter-day relative errors expressed by precision (C.V.) of the individual calibrators for the KC 12291 in plasma were ≤9 and 14%, respectively. The intra- and inter-day accuracy of each calibrator of KC 12291 in plasma was between 94.1 and 104% and 94.1 and 103.1%, respectively. The intra- (y= 4.589E−03x−0.0409, r^2=0.9999) and inter-day (y=4.678E−3x−0.0202, r^2=0.9997) mean regression equations were linear in the 10–1000 ng/ml urine concentration range for KC 12291. The intra- and inter-day precision (C.V.) of the individual calibrators for the KC 12291 in urine were ≤14 and 13%, respectively. The intra- and inter-day accuracy of each calibrator of KC 12291 in urine was between 87.5 and 96.0% and 81.4 and 102.8%, respectively. In Fig. 2, a peak appeared with the retention time of KC 13194 after oral dosing. The concentration of KC 13194 was determined in the way as KC 12291 in plasma. Resolution between KC 12291 and the I.S. as well as KC 13194 and the I.S. were 2.6 and 3.8, respectively. Resolution between KC 12291 and the I.S. in urine was 2.5. The metabolite, KC 13194 was not detectable in plasma after intravenous treatment. The mean regression equation (y= 0.9533x+0.2137, r^2=0.9997) obtained from the five replicate curves of KC 13194 in plasma in a day was linear. The intercepts relative to the response of the highest calibration point of KC 12291 and KC 13194 in plasma and urine were between ±2%. The lowest calibration level used in the validation calibration curves of KC 12291 yielded a concentration of 10 ng/ml in plasma and urine. This was set to a limit of quantification that can be measured with acceptable precision (≤15.95%) in plasma and (≤7.47%) in urine, respectively. The accuracy of the lowest point of the calibration curves of KC 12291 was between 81.3 and 116.7% in plasma and between 91 and 100.2% in urine. The upper limit of quantification of KC 12291 was determined using the highest level of calibration curves yielding concentrations 1000 and 2000 ng/ml in plasma and urine, respectively. This concentration was measured with an acceptable precision (C.V.) of 10.3% in plasma and 6.0% in urine. The accuracy of this concentration was between 96.2 and 115.0% in plasma and between 91 and 108% in urine. The lower and upper limits of quantification of KC 13194 in plasma were the lowest and highest calibration levels, (100 and 2500 ng/ml) with acceptable precision (4.5 and 5.0%, respectively). At these concentrations the accuracy was between 116.6 and 104.9%.

3.3. Precision and accuracy of the assay

The intra-day precision and accuracy of the method were determined by analyzing six aliquots of the validation samples of KC 12291 and KC 13194 in plasma and of KC 12291 in urine. The mean value of intra- and inter-day precision (C.V.) of the validation samples of KC 12291 in plasma and urine was less than 13% and the accuracy was between 105 and

115%, respectively. The intra-day precision of validation samples of KC 13194 in plasma was ≤5.0% and the accuracy was between 83.8 and 89.7%.

3.4. Recovery

The absolute recovery was determined in plasma and urine at each calibration point of KC 12291 during three validation runs. The recovery was determined by dividing the ratio of peak areas of extracted KC 12291 and the non-extracted I.S. versus the ratio of the peak areas of non-extracted KC 12291 and I.S. The result was multiplied by 100. The recovery for KC 12291 at each concentration point ranged from 61 to 71.6% in plasma and from 82.5 to 106.1% in urine, respectively.

3.5. Stability

The stability of KC 12291 in plasma and urine was determined at −20°C, at concentrations of 30 and 900 ng/ml and 60 and 1800 ng/ml in plasma and urine, respectively, by comparing the values of the mean of the respective sample with an initial value at various intervals (from 1 to 8 weeks). Three samples from each concentration group were analyzed.

The data generated on weeks 1 and 8 revealed no significant change in KC 12291 concentrations in plasma and urine. For the determination of the stability of the sample preparation, the lowest and the highest validation samples were thawed and frozen in three cycles. Three freeze–thaw cycles did not significantly alter the amount of KC 12291 analyzed in the spiked plasma and urine samples. The precision (C.V.) of the validation samples ranged from 1.61 to 14.66% in plasma and from 2.73 to 11.33% in urine. The accuracy was between 83.6 and 109.6% in plasma and between 98.62 and 114.98% in urine, respectively.

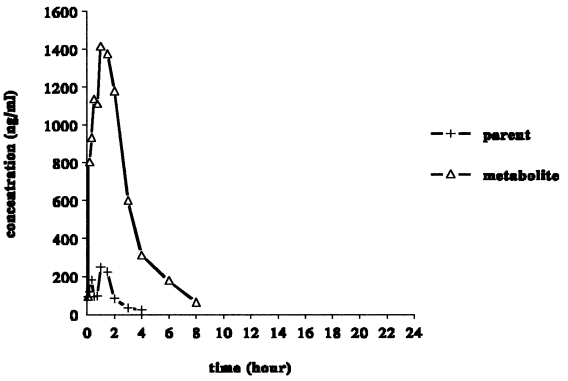

Fig. 3. Plasma KC 12291 and KC 13194 concentrations in male rats (n=5) following a single oral dose of 60 mg/kg body weight of KC 12291.

3.6. Ruggedness (column to column)

The linearity (y=4.1157E−03x−0.01412, r^2= 0.9998) of the calibration curves of KC 12291 was determined on another column of the same quality but different serial number in plasma in a day (n=5). The value of the mean intercept of the regression line (b value) fell within the range ±2% of the detector response obtained at the highest calibration point. The mean precision (C.V.) of validation samples was ≤5% and accuracy at the different concentrations was between 112.0% and 115.46% (n=5) in plasma in a day.

4. Application

4.1. Plasma

The validated method was used to analyze more than 700 plasma samples from pharmacokinetic studies following intravenous and oral administrations of KC 12291 to male and female rats. Calibration levels and quality control samples were identical to the calibration levels and validation samples used during the validation assay. The samples were analyzed during an 8-week period in 12 sequences. The values obtained for the calibration levels were in a similar range to that observed during the validation. In each case the correlation coefficient (r^2) was above 0.997. Plasma levels of KC 12291 and its main metabolite, KC 13194 could be followed up 6–8 h post intravenous drug administration and 12–24 h post oral drug administration. After an oral dosing significant differences were found between the plasma concentrations of the parent compound and its main metabolite, KC 13194 in males

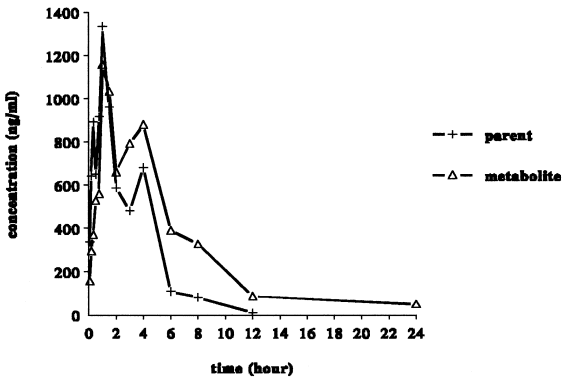

Fig. 4. Plasma KC 12291 and KC 13194 concentrations in female rats (n=5) following a single oral dose of 60 mg/kg body weight of KC 12291.

and females, respectively (Figs. 3 and 4). The pharmacokinetic results will be published elsewhere.

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