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Full Length Research Paper

Determination of strontium in rat plasma and plasma ultrafiltrate by Zeeman Furnace atomic absorption spectroscopy and its application to a pharmacokinetic study

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A rapid and sensitive analytical assay for the determination of total strontium concentrations in rat plasma was developed and validated. The total strontium levels were determined by use of graphite-furnace atomic-absorption spectrometry (GF-AAS) with Zeeman correction, 1000°Ci n ashing temperatures and 2700°C in atomization temperatures after 3.5% HNO_3 protein precipitation. The accuracy was between 95.46 and 102.24% (intra-day variation), and 101.74 and 102.15% (inter-day variation). The precision was between 1.86 and 8.24% (intra-day variation), and 1.27 and 11.20% (inter-day variation). Linear calibration curves were obtained over the concentration range 0.2 to 10 µg/ml(r = 0.9993). Validation data were within the acceptable limits for analytical methods. This assay was successfully applied to evaluate the pharmacokinetics of strontium fructose 1, 6-diphosphate (Sr-FDP), a new compound in rats and free strontium after plasma ultrafiltrate was also performed by direct determination. Significantly different pharmacokinetics between total and free strontium were observed that provided more information for the pharmacokinetic study of Sr-FDP.

Key words: Graphite furnace atomic-absorption spectrometry (GFAAS), strontium fructose 1, 6-diphosphate, strontium, ultrafiltrate, anti-osteoporosis.

INTRODUCTION

Strontium (Sr) is recognized as an essential trace element in biological and medical fields. It is found in soft tissues, blood and bones. Many studies have shown that Sr^{2+} can stimulate bone formation and inhibit bone resorption both *in vitro* and *in vivo* (Ammann, 2005; Marie, 2005a, 2006), and has potential benefit on the osteoporosis treatment (Marie, 2005b). Besides, stable strontium as a tracer has been used to estimate intestinal absorption of calcium in biological and clinical research for a long time due to a close correlation between the intestinal absorption of calcium and strontium (Sips et al., 1994; Wasserman, 1998; Dijkgraaf-Ten et al., 2000). Previously, the radioactivity of strontium such as ^{90}Sr and ^{89}Sr has been widely used as tracers in the study of strontium and bone metabolism (Cappadona and Dardanoni, 1962; Petrow, 1965). However, the use of radioactive substances in preclinical and clinical research is disadvantageous due to the costs of radionuclides, the disposal of the radioactive wastes and the potentially hazardous effect of radionuclides to animal and human subjects.

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In recent years, some new techniques including graphite furnace atomic absorption spectrometry (GFAAS) (Barto et al., 1995), flame atomic emission spectroscopy with a filament vaporizer (FAAS) (Leeuwenkamp et al., 1989), inductively electrothermal atomization absorption spectrometry (ETAAS) (Scancar et al., 2000), inductively coupled plasma mass spectrometry (ICP-MS) (Vandecasteele et al., 1990; Raffalt et al., 2008), etc. have been carried out for analyzing stable Sr in the environmental, biological and pharmaceutical matrices. Among them, FAAS in general is not sensitive enough to directly determine the metal element at the relatively low concentration ranges as it naturally occurs in biological fluids (Leeuwenkamp et al., 1989). Moreover, in the analysis of biological material, FAAS-based methods are known to be subject to several chemical interferences, which will cause analysis error. The effects (signal suppression and interferences) caused by the complex matrix of a biological sample, have to be taken into account in case of ICP-MS (Chrastny et al., 2006; Chen et al., 2007). GFAAS is the preferred technique since it requires low sample volume, provides adequate sensitivity and low limit of detection. Moreover, graphite furnace atomic absorption spectrometry with Zeeman background correction has the matching intensity with regard to the wavelength of the sample beam and the reference beam (for background deduction) could eliminate interference coexistence material interference (Shi-Lian et al., 1987).

Strontium fructose 1, 6-diphosphate (Sr-FDP), a new anti-osteoporosis compound, is undergoing preclinical evaluation in our institute, Figure 1. To obtain accurate *in vivo* information, it is pivotal to establish and validate a simple and sensitive assay. The present study was designed to detect total strontium concentration after optimization of protein precipitation and atomization temperature by a specific GFAAS with Zeeman background correction. The validated method was applied to characterize the pharmacokinetic behavior of Sr after a single oral gavage dose of Sr-FDP in rat.

Analysis of Free strontium in plasma ultrafiltrate was performed simultaneously by direct determination using the same instrumental method. Comparison of the fate of total and free Sr in rat may provide more helpful information for the preclinical and clinical pharmacokinetic study.

MATERIALS AND METHODS

Reagents and chemicals

Atomic absorption standard for strontium (0.1 mg/ml) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); nitric acid, perchloric acid and trichloroacetic acid (TCA) were guaranteed reagent was from Shanghai Jingchun Chemical Reagent Corporation (Shanghai, China); Sr-FDP was obtained from National Center for Biochemistry (Nanjing, China); purified water was produced by a Milli-Q system (Millipore, MA, USA).

Experimental animals

Sprague–Dawley specific-pathogen-free (SPF) rats (body weight 200 ± 10 g) obtained from the Animal Center of Nanjing Medical University was used in this study. Rats were housed under controlled conditions including a room temperature of 22 ± 1°C and fed with standard solid food and deionized water *ad libitum* during the whole experimental period. The experimental procedures including the raising, feeding, and the whole manipulative process were approved by the Animal Ethical Committee and followed the guidelines of the Animal Ethics Committee of Nanjing University of Technology.

Instrumentation

The analyses were performed with a VARIAN AA240Z Zeeman graphite-furnace atomic absorption spectrometer equipped with a 10 partition tubes (PART NO: 63-100012-00,BATCH NO: 101651776-42761-88108), a PSD 120 Programmable Sample Dispenser, and pyrolytically coated graphite-furnace tubes. The light source was a hollow cathode lamp, which was used at 25 mA at 460.7 nm. Nitrogen were alternately used as purge gasses (300 ml/min, during atomization 0 ml/min), and 10 µL sample aliquots were injected during the determination. Each sample was determined three times repeatedly. All automated pipettes used were “Finnpipettes” (Labsystems, Helsinki, Finland) equipped with disposable polypropylene pipette tips. The optimum instrumental conditions for elementary analysis are showed in Table 1.

Total plasma and plasma ultrafiltrate processing

Plasma 20 µL was mixed with 80 µL 3.5% HNO_3 by vortex for 3 min and then centrifuged at 12,000 rpm for 10 min. The supernatants were diluted by 0.2% HNO_3 to 1000-fold, and volumes of 10 µL were injected into the atomizer for total plasma analyses. Each sample was analyzed three times repeatedly. An ultrafiltration method was employed to measure the free strontium concentration in the rat plasma. Whole blood samples were collected in 1.5 ml heparin containing Eppendorf tube 1.5 and plasma was separated by centrifuge. Then ultrafiltration was immediately performed by centrifuging the total plasma through an ultrafiltrate filter (Millipore, Tokyo, Japan), molecular weight cutoff of 30 KDa, and for 30 min (3,000 rpm, 4°C). All samples were stored at -80°C until analysis. Before analysis samples of ultrafiltrate were also diluted by 0.2% HNO_3 to 1000-fold as earlier described.

Standard-addition working curve and the limit of detection (LOD)

A Sr stock solution containing 2, 5, 10, 20, 50 and 100 µg/ml was made by dissolving 100 µg/ml atomic absorption standard for strontium with 0.2% HNO_3. Subsequently, different concentration stock solution were pipetted respectively in 20 µL pooled plasma, then 80 µL 3.5% HNO_3, were added to each of them, vortexed for 3 min and centrifuged at 12,000 rpm for 10 min. The supernatants were diluted 1000-fold by adding 0.2% HNO_3, and volumes of 10 µL were injected into the atomizer for analyses. Through a series of dilution, the Sr concentrations of the samples from the pharmacokinetic studies came within the linear range of the calibration curve. The limit of detection (LOD) was calculated by using the Equation of LOD = 3 × SBL / b, where SBL is the standard deviation of 10 consecutive measurements of the blank solutions and b is the slope of the calibration curve. In this study, in order to obtain the SBL, the absorbances of 0.2% HNO_3 were measured 10 times during the analysis process from batch to batch.

Table 1. Instrument settings and furnace conditions for plasma strontium determination.

Spectrophotometer	VARIAN AA240Z Zeeman graphite-furnace atomic absorption spectrometer
Wavelength	460.7 nm
Slit width	0.5 nm
Signal measurement	Peak height
Reading cycle	2.9 s

Phase	Temperature(℃)	Ramp time(s)	Gas flow(ml/min)
Drying	85	15	300
	95	40	300
	120	10	300
Charring	1000	5	300
	1000	1	300
Gas-stop	1000	2	0
atomization	2700	0.9	0
	2700	2	0
Burn-out	2800	2	300

Precision and accuracy

The intra- and inter-day accuracy and precision of this method was carried out by spiking with known concentration levels of strontium (0.5, 2 and 10 µg /ml) to plasma before deproteinization. At least five replicates of each point were analyzed to determine the intra-day accuracy and precision. This process was repeated on five consecutive days in order to determine the inter-day accuracy and precision. The equation of intra- and inter-day assay accuracies was calculated as; accuracy (%) = [Csr(t) - Csr(e)] / Csr(s) × 100, where (Csr(t) = total strontium in plasma; Csr(e) = endogenous strontium in plasma and Csr(s) = spiking with known concentration levels of strontium). Precisions of assay were expressed using the coefficient of variation (C.V.) between assays.

Extraction recovery and choice for precipitant

Extraction recovery was evaluated at concentrations of 0.5, 2 and 10 µg/ml in different sample matrices. Each set of samples was analyzed five times. The samples used were; A: standards extracted from spiked plasma; B: standards extracted from blank plasma; and C: standards in the pure solution. Plasma samples were treated as described in section “Total plasma and plasma ultrafiltrate processed.” The extraction recovery was determined by comparing the absorbance of set (A and B) to the absorbance of set C. To select a suitable precipitant for deproteinization with maximal extraction recovery, plasma sample were treated via 3.5% HNO_3, 8% TCA and 2.4% $HClO_4$, respectively and the absorbances were compared.

Application of the method to pharmacokinetic study

After an overnight fast, the rats (n = 6) were orally dosed with 220 mg/kg of FDP-Sr and the other group rats (n = 6) for baseline considered endogenous strontium were orally dozed with deionized water. Blood samples were collected at 0 (predose), 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 h after administration. Then the samples were placed in heparinized Eppendorf and plasma was immediately separated by centrifugation at 3000 rpm for 10 min and aliquoted into two parts. One part was used to determine total strontium in plasma, and the other part was used as ultrafiltrate to determine free strontium analysis. All samples were stored at –80℃ prior to analysis.

Statistical analysis

A non-compartmental model by using DAS (Drug And Statistics) 2.0 soft (Mathematical Pharmacology Professional Committee of China, Shanghai, China) was used to calculate pharmacokinetics parameters. Data were expressed as mean ± S.D. Student’s t-test was used to assess statistical significance and significance was determined as $P < 0.05$.

RESULTS AND DISCUSSION

Atomization temperature optimization

The furnace program was finalized after optimization of drying, ashing, and atomization temperatures for Sr determination. Optimal ashing temperature and atomization temperature curves of strontium in plasma are shown in Figure 2. The maximum ashing temperature observed at 1000℃ was superiority, which ensured strontium in the plasma could not be atomized simultaneity.

In additional, the low atomization temperature for this study would prolong the graphite tube lifetime. In con-sequence, 1000 and 2700℃ was chosen as optimal ashing temperature and atomization temperature, respectively which were lower than that of previous report. This was found to be cost effective as each tube could be used more than 200 times. After atomization, a burn-out

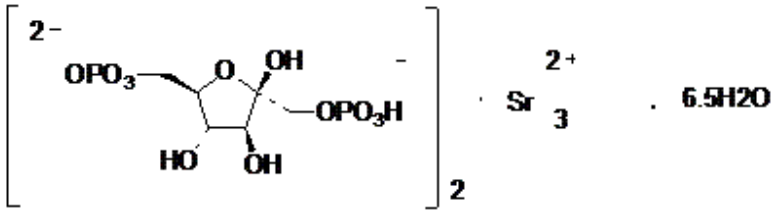

Figure 1. Chemical structures of strontium fructose 1, 6-diphosphate.

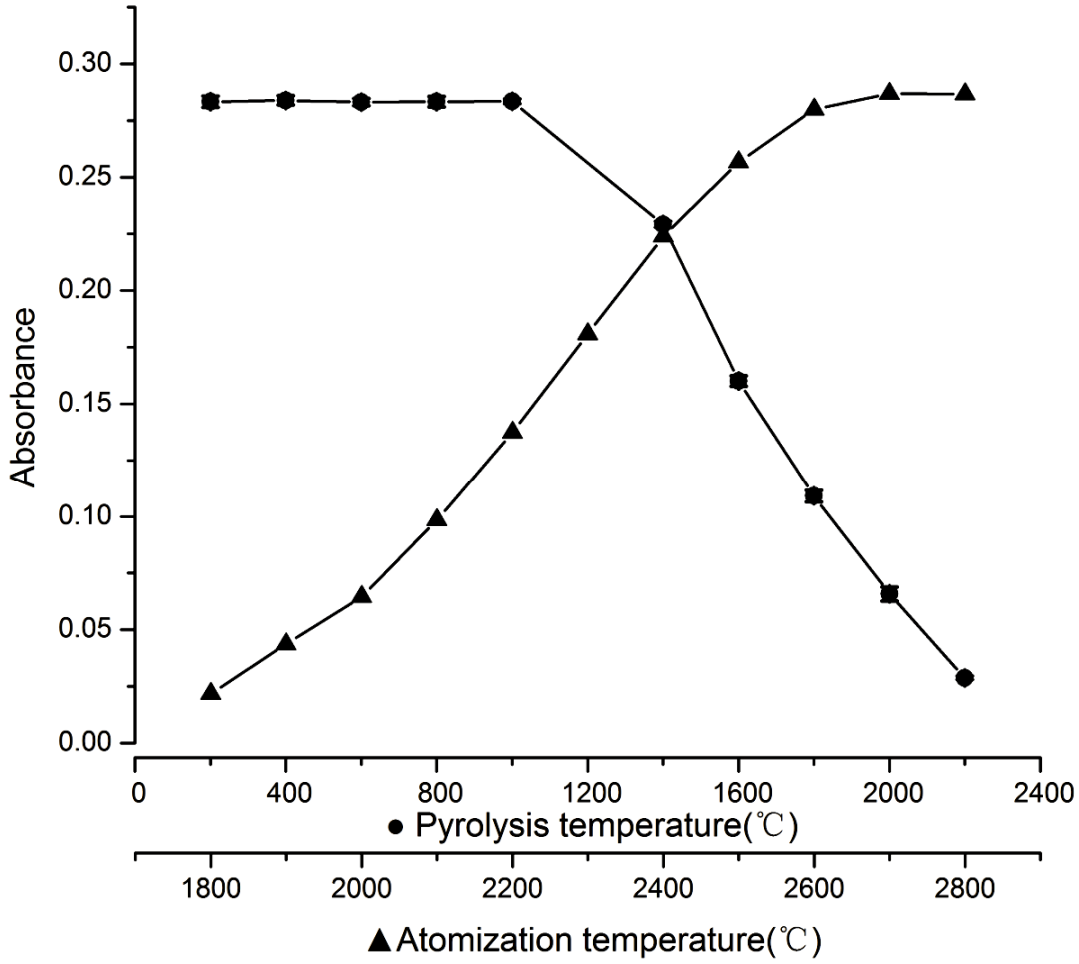

Figure 2. Ashing and atomization temperature curves of strontium by GFAAS.

temperature of 2800℃ for 2s appeared to be necessary to avoid any carryover between two injections. Together, all the steps required 77.9 s for analyzing 10 µL sample, for one injection.

Extraction recovery

An extraction recovery sample of strontium at 0.5, 2 and 10 µg/ml concentrations was obtained after deproteinization by 3.5% HNO_3, 8% TCA and 2.4% $HClO_4$ (n = 5). Supernatant of each sample was cleared after centrifuging. According to "2.4" method described in sample treatment, the absorbance was determined. The extraction recovery results of 3.5% HNO_3, 8% TCA and 2.4% $HClO_4$ are presented in Table 2. Our result indicates that 3.5% HNO_3, 8% TCA and 2.4% $HClO_4$ had good extraction recovery and meet current guidelines for bioanalytical methods. However, strontium signals at 2 µg/ml concentration were significantly increased in both 8% TCA and 2.4% $HClO_4$ compared to 3.5% HNO_3 (Table 3). It suggested that chlorine of TCA or $HClO_4$ would

Table 2. Intra- and inter-day assay precision and accuracy for GFAAS assay of strontium in rat plasma (n = 5).

Nominal concentration (µg/ml)	Intra-day			Inter-day		
	Measured concentration (µg/ml) (mean ± SD)	C.V. (%)	Accuracy (%)	Measured concentration (µg /ml) (mean ± SD)	C.V. (%)	Accuracy (%)
0.5	0.51 ± 0.04	7.87	102.24	0.51 ± 0.03	6.70	102.15
2	1.91 ± 0.16	8.24	95.46	2.04 ± 0.23	11.20	102.05
10	10.10 ± 0.19	1.86	101.03	10.25 ± 0.13	1.27	101.74

Table 3. Extraction recovery for GFAAS assay of strontium in rat plasma (n = 5).

Extraction solvent	Nominal concentration (µg/ml)	Extraction recovery (%)
0.5% HNO_3	0.5	64.71 ± 0.03
	2	75.11 ± 0.10
	10	88.67 ± 0.21
8% TCA	0.5	58.48 ± 0.05
	2	68.94 ± 0.07
	10	84.72 ± 0.22
2.4% HClO4	0.5	53.58 ± 0.07
	2	71.07 ± 0.08
	10	86.73 ± 0.17

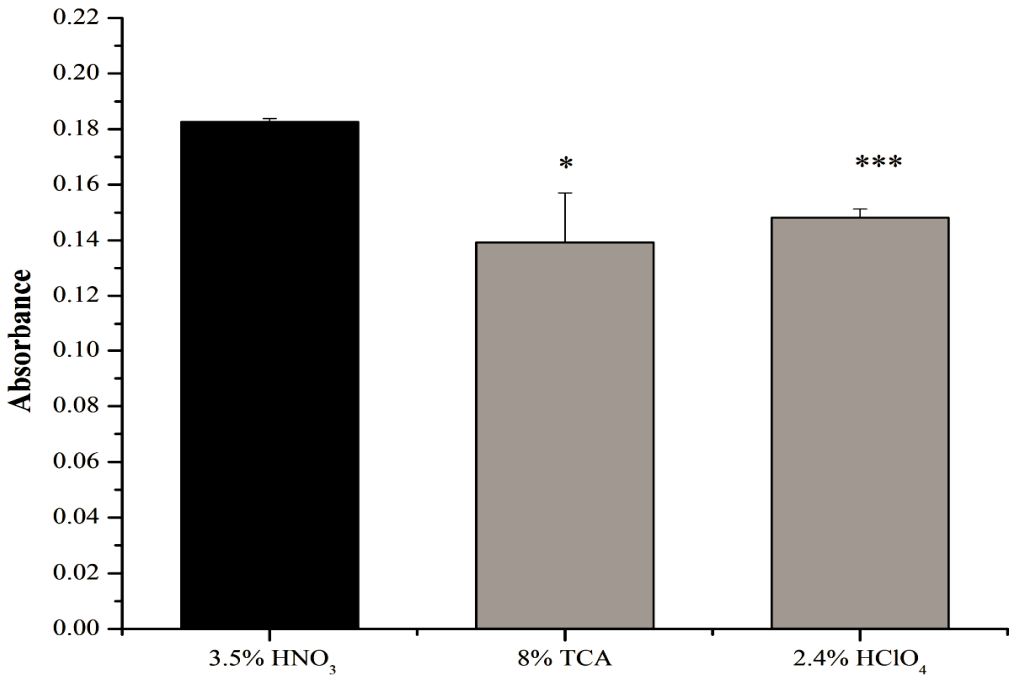

Figure 3. Strontium signals in different kinds of protein precipitants. Results are represented mean ± S.D. of triplicate samples with three repeated determinations for each sample. Values with a superscript are significantly different from those of 3.5% HNO_3 (*P<0.05; **P<0.01; ***P<0.001).

interfere with response of the determination of trace strontium by GFAAS.

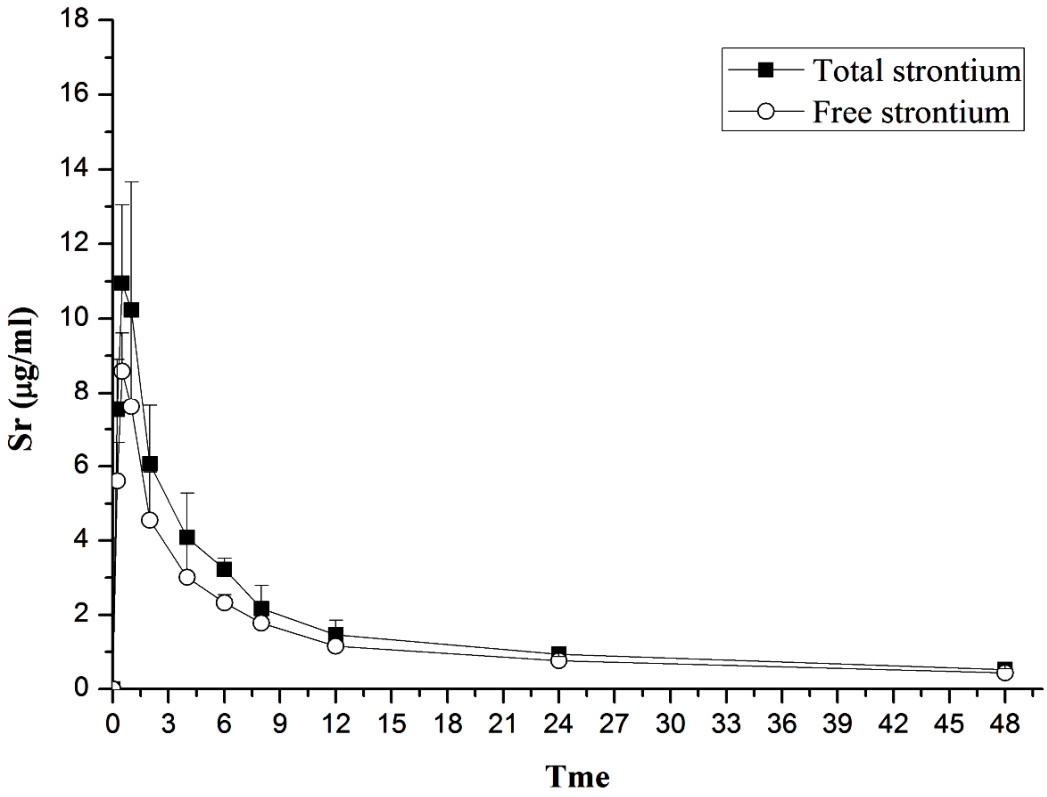

Figure 4. Concentration–time curves generated for total and free Sr in the plasma of Sprague–Dawley rats given a single i.g. administration of 220 mg/kg Sr-FDP. Each point represents the mean ± SD of six independent experiments.

Precision and accuracy

Strontium plasma samples at three concentration levels (0.5, 2 and 10 µg/ml) were analyzed for accuracy and precision. Assay performance data for strontium are listed in Table 2. For the devised assay, intra-day C.V. was 7.87, 8.24 and 1.86%, while inter-day C.V. was 6.70, 11.20 and 1.27%. Also, intra-day accuracy ranged from 95.46 to 102.24% and inter-day accuracy from 101.74 to 102.15%. The precision and accuracy were thus in accordance with acceptable criteria for bio-analytical methods.

Standard-addition working curve and the limit of detection (LOD)

Calibration curve of strontium was Y = 0.0253X + 0.019 and correlation coefficients were 0.9993. Standard deviation of blank was 0.0014 calculated by the absorbance of 10 consecutive measurements of 0.2% HNO_3. LOD = 3 × SBL was found to be 0.17 µg/ml. In additional, the endogenous concentrations of Sr measured in plasma of six rats were 0.372 ± 0.149 µg/ml. So the LOD of this method could meet the testing requirements.

Pharmacokinetic study

The presented method was applied to quantify strontium in the plasma of six rats following a single 220 mg/kg oral administration dose. The plasma strontium concentration of each sampling time point was corrected by deducting the endogenous concentration from the totally measured concentration. The concentration versus time profile is shown in Figure 4 and pharmacokinetic parameters are listed in Table 4. For total strontium, the mean maximum plasma concentration (C_{max}) of Sr-FDP was 11.92 ± 2.11 µg /ml occurring at 0.75 ± 0.27 h post dose. The mean elimination half-life ($t_{1/2}$) was 21.51 ± 2.94 h and area under the plasma concentration versus time curve ($AUC_{0-\infty}$) was 95.21 ± 14.79 mg/L*h. Similar to total strontium, a peak plasma level for free Sr of 9.18 ± 1.44µg /ml was reached immediately within 1 h, and no statistics distinction was observed in $t_{1/2}$(21.08 ± 3.68 h), V(81.7 ± 1.89 L/kg) and CL(2.67 ± 0.24 L/h/kg) when compared with total strontium. Although, high free strontium was found in plasma due to poor plasma protein binding (Reginster, 2002), significant differences of Cmax, AUC0-t and AUC0-∞ were observed in between total and free strontium.

Table 4. The main pharmacokinetic parameter of total and free strontium after single oral dose of 220 mg to rats (mean ± S.D, n = 6).

Pharmacokinetic parameter	Total strontium	Free strontium
$T_{1/2}$ (h)	21.51 ± 2.94	21.08 ± 3.68
Tmax (h)	0.75 ± 0.27	0.75 ± 0.27
Cmax (mg/L)	11.92 ± 2.11	9.18 ± 1.44*
CL (L/h/kg)	2.36 ± 0.39	2.67 ± 0.24
V(L/kg)	72.85 ± 13.15	81.7 ± 1.89
AUC(0-t) (mg/L*h)	78.82 ± 10.45	62.09 ± 6.07*
AUC(0-∞) (mg/L*h)	95.21 ± 14.79	75.25 ± 6.26*
MRT (h)	23.86 ± 5.14	24.49 ± 5.13

Values with a superscript are significantly different from those of the total strontium (*P<0.05).

Conclusion

So far, we described a rapid and convenient method for the determination of total and free strontium levels in rat plasma. Compared with previously described methods, 3.5% HNO_3 was chosen as a precipitant due to good signal intensities eliminating the interference of chloride and decrease depletion of the graphite tube before analysis by GFAAS. The optimization of atomization temperature may further prolong the graphite tube lifetime. What's more, this method was readily applicable to the determination of strontium in plasma samples with high sensitivity, precision, accuracy and low cost according to generally accepted criteria and it was successfully applied to pharmacokinetic studies of Sr-FDP.

Finally, Sr-FDP, a novel anti-osteoporosis drug candidate, was researched for pharmacokinetic characteristic of total and free strontium in rat, respectively. For the pharmacokinetic study of most Sr complex compounds, only total Sr level was measured in plasma to obtain PK parameters (Barto et al., 1995; Sips et al., 1996), though it was known that the free form of Sr played its role in the treatment. Our current study showed that significant differences of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ indeed existed between total and free strontium *in vivo* after dosing Sr-FDP. This also indicated that more attention should be paid to the free drug concentration in the pharmacokinetic study, especially for the drug with high protein combination. In addition, this method could also be used to determine other ions by simultaneously measuring total and free drug concentration to scientifically evaluate pharmacokinetics characteristics and avoid to toxicity.

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