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(54) **Title:** THE QUANTITATIVE DETERMINATION OF RISEDRONATE IN URINE BY SPE-LC-MS-MS

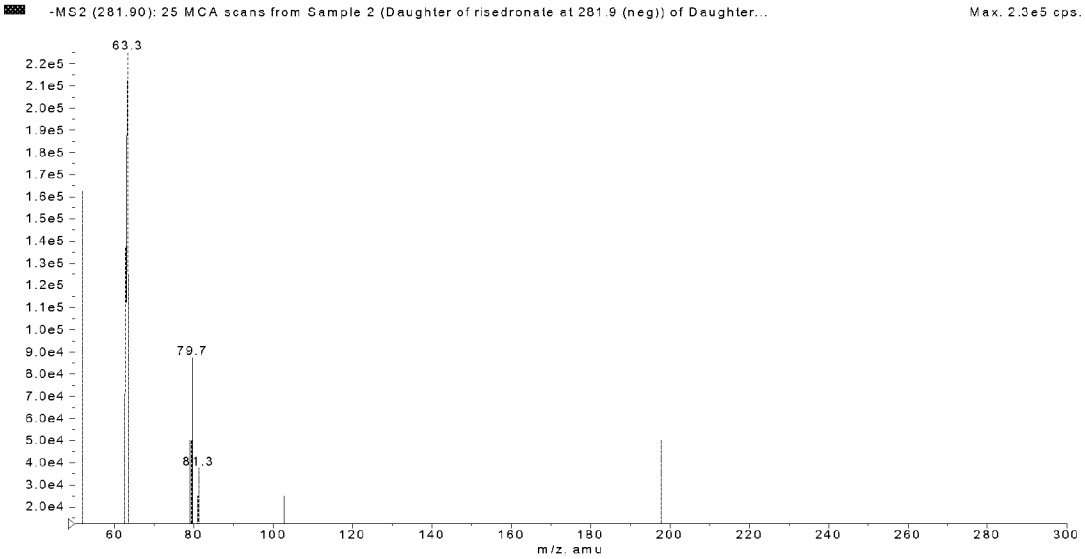

FIGURE 1. ESI-MS Spectrum of Risedronate

(57) **Abstract:** The present invention is directed to a SPE-LC-MS-MS method for quantitatively determining risedronate in a urine sample.

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THE QUANTITATIVE DETERMINATION OF RISEDRONATE IN URINE BY SPE-LC-MS-MS

FIELD OF THE INVENTION

The present invention is directed to a SPE-LC-MS-MS method for quantitatively determining risedronate in a urine sample.

BACKGROUND OF THE INVENTION

Bisphosphonates inhibit bone resorption and are effective treatments for metabolic bone diseases including osteoporosis and Paget's disease (I. Elomaa, C. Blomqvist, L. Porkka, T.Holmstrom, T.Taube, C.Lamberg-Allardt, G.H. Borgstrom. *Lancet* 1985; i:1155; J.P. Kosonen, *J. Pharm Biomed Anal.* 1992; 10:881; J.A. Cantril, H.M. Buckler, D.C. Anderson, *Ann. Rheumatol. Dis.* 1986; 45:1012; H.Fleisch, *Horm. Metab. Res.* 1997; 29:145). The prevention of bone resorption results from inhibitory effects on the function of mature osteoclasts. Several bioanalytical methods have been published for bisphosphonates. In general, those methods are mainly based on ion-exchange and ion-pair chromatography with UV, fluorescence (with a pre or post column derivatization), conductivity, flame photometric phosphorus selective, refractive index and explorative light-scattering detection (V. Virtanen, L.H.J. Lajunen, *J. Chromatogr.* 1993; 617:291; V. Virtanem, L.H.J. Lajunen, *Talanta* 1993; 40: 661; S.E. Meek, D.J. Pietrzyk *Anal. Chem.* 1988; 60:1397; M.J. Lovdahl, D.J. Pietrzyk, *J. Chromatogr.* A 1999; 850:143; R. Niemi, H. Taipale, M. Ahlmark, J. Vepsalainen, T. Jarvinen, *J. Chromatogr.* B 1997; 701:97; T.L. Chester, *Anal. Chem.* 1980; 52:1621).

The technique of GC-MS, combined with acylation and silylation, has been used to determine Risedronate in human urine (D.Y. Mitchell, R.A. Eusebio, L.E. Dunlap, K.A. Pallone, J.D. Nesbitt, D.A. Russell, M.E. Clay, P.J. Bekker, *Pharm. Res.* 1998; 15:228). The more sensitive method for analysis of Risedronate in human urine has been achieved using enzyme linked immunosorbent assay (ELISA) (D.Y. Mitchell, M.A. Heise, K.A. Pallone, J.D. Nesbilt, M.E. Clay, J.D. Nesbitt, D.A. Russell, C.W. Melson, *J. Clin. Pharmacol.* 1999; 48:536). The column-switching ion-pair HPLC with UV detection has also been reported to quantify the Risedronate in human urine (P.T. Vallano, S.B. Shugars, W.F. Kline, E.J. Woolf, B.K. Matuszewski, *J. Chromatogr.* B 1003; 794:23). Although some of these methods showed a very high sensitivity, they are all very complicated and time-consuming.

Recently, more effort has been put on the development of LC/MS/MS method for risedronate with post-extraction or on cartridge derivertization (L.S. Zhu, V.N. Lapko, J.W.

Lee, Y.J. Basir, C. Kafonek, R. Olsen, C. Briscoe, Rapid commun. Mass Spectrom. 2006, 20: 3421; S.Turcotte, J. Couture, F. Vallee, AAPS PharmSci Vol. 5, No. 4, Abstract M1357 (2003).

SUMMARY OF THE INVENTION The present invention is directed to a method for quantitatively determining risedronate in a urine sample comprising:

a) adding an internal standard to the urine sample;
b) applying the urine sample to an Oasis HLB cartridge, wherein the cartridge has been pre-conditioned with methanol;
c) washing the cartridge with about 1% (v/v) TEA in water and about 1% (v/v) formic acid in methanol;
d) eluting risedronate, at least once, with a mixture of about 60% (v/v) methanol and about 40% (v/v) water containing about 3 mM EDTA under vacuum;
e) evaporating the eluted solution and reconstituting with a mixture of 10% (v/v) methanol and 90% (v/v) 0.05 M NH_4Ac-NH_4OH buffer to provide a sample mixture of risedronate and the internal standard; and
f) analyzing the sample mixture with a LC-MS/MS system.

The aforesaid SPE-LC-MS-MS method for quantitative determination of risedronate in a urine sample is relatively simple, sensitive, precise and accurate, and more fully discussed with the aid of the following figures and detailed description below.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 is the ESI-MS Spectrum of Risedronate.

FIGURE 2 is the ESI-MS Spectrum of Deoxy-risedronate.

FIGURE 3 is the Chromatogram of Risedronate in Mouse Urine (LLOQ 10 ng/mL), wherein the intensity of the peak at retention of 2.27 minute is more than three times of the intensity of the same peak in the HPLC Spectrum of Blank Mouse Urine Sample. This means that the risedronate concentration at 10 ng/mL in mouse urine can be quantified.

FIGURE 4 is the Chromatogram of Mouse Control Urine.

FIGURE 5 is the Typical Chromatogram of Risedronate and Deoxy-Risedronate.

FIGURE 6 is the Typical Calibration Curve of Risedronate in Mouse Urine.

DETAILED DESCRIPTION OF THE INVENTION

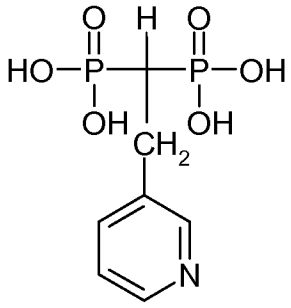
Definitions and Abbreviations

As used above, and throughout the description of the invention, the following abbreviations, unless otherwise indicated, shall be understood to have the following meanings:

amu	Atom mass unit
Cps	Count per second
CV%	Percent coefficient of variation
Diff%	Percentage difference between theoretical concentration and measured concentration
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrical Spray Ionization
HPLC	High Pressure Liquid Chromatography
LC-MS	High Pressure Liquid Chromatography-Mass Spectrometry
LLOQ	Lower limit of quantity
MRM	Multiple reaction monitoring
MS	Mass Spectrometry
m/z	Mass/charge
NH_4Ac	Ammonium acetate
NH_4OH	Ammonium hydroxide
SD	Standard deviation
SPE	Solid phase extraction
TEA	Triethylamine
v/v	Volume/volume

As used above, and throughout the description of the invention, the following terms, unless otherwise indicated, shall be understood to have the following meanings.

"Deoxy-Risedronate" means (1-phosphono-2-pyridin-3-yl-ethyl)-phosphonic acid, having the following chemical structure:

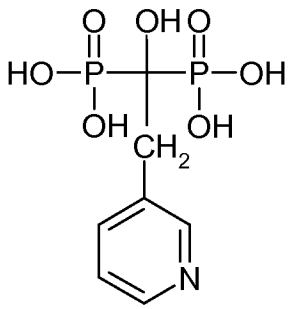


"Inertsil Phenyl-3 HPLC column" is manufactured and sold by Varian, Inc.

"Internal standard" is a chemical substance that is added in a constant amount to samples, blank and calibration standards in an analysis. This chemical substance is used for calibration by plotting the ratio of the analyte signal to the internal standard signal as a function of the analyte concentration of the standards. This is done to measure and correct for the loss of analyte during sample preparation or sample inlet. Particularly, the internal standard is a compound that has similar, but not same, chemical structure to the analyte.

"Oasis HLB cartridge" is manufactured and sold by Waters Corporation. The size of the cartridge is dependent upon the risedronate concentration in the urine sample. The lower risedronate concentration the urine sample has, the bigger size of the cartridge, and the larger volume of urine sample and the solution to wash the cartridge and elute risedronate should be used.

"Risedronate" means (1-hydroxy-1-phosphono-2-pyridin-3-yl-ethyl)-phosphonic acid, having the following chemical structure:



"Urine sample" means mammalian urine sample, including human urine sample.

Particular Embodiment of the Invention

One particular embodiment of the invention is the method for quantitatively determining risedronate in a urine sample, wherein the urine sample is a mouse or rat urine sample.

Another particular embodiment of the invention is the method for quantitatively determining risedronate in a urine sample, wherein the internal standard is deoxy-risedronate.

Another particular embodiment of the invention is the method for quantitatively determining risedronate in a urine sample, wherein the Oasis HLB cartridge is a 30 mg Oasis HLB catridge.

Another particular embodiment of the invention is the method for quantitatively determining risedronate in a urine sample, wherein the internal standard is deoxy-risedronate that is added as a solution in water; more particularly as an about 10 μg/mL solution in water.

Another particular embodiment of the invention is the method for quantitatively determining risedronate in a urine sample, wherein the amount of the urine sample is about 0.4 mL.

Another particular embodiment of the invention is the method for quantitatively determining risedronate in a urine sample, wherein the cartridge has been pre-conditioned with about 1 mL of methanol.

Another particular embodiment of the invention is the method for quantitatively determining risedronate in a urine sample, wherein the cartridge has been pre-conditioned with about 1 mL of methanol and followed by about 0.5 mL of 1% (v/v) TEA in water.

Another particular embodiment of the invention is the method for quantitatively determining risedronate in a urine sample, wherein the cartridge is washed with about 0.5 mL of about 1% (v/v) TEA in water and about 0.5 mL of about 1% (v/v) formic acid in methanol.

Another particular embodiment of the invention is the method for quantitatively determining risedronate in a urine sample, wherein the amount of the mixture of about 60% (v/v) methanol and about 40% (v/v) water containing about 3 mM EDTA is about 1.5 mL.

Another particular embodiment of the invention is the method for quantitatively determining risedronate in a urine sample, wherein risedronate is eluted twice with about 0.75 mL of the mixture of about 60% (v/v) methanol and about 40% (v/v) water containing about 3 mM EDTA.

Another particular embodiment of the invention is the method for quantitatively determining risedronate in a urine sample, wherein the amount of the mixture of 10% (v/v) methanol and 90% (v/v) 0.05 M NH_4Ac-NH_4OH buffer is about 100 μL.

Another particular embodiment of the invention is the method for quantitatively determining risedronate in a urine sample, wherein the internal standard is deoxy-risedronate, and analyzing the sample mixture with a LC-MS/MS system comprises separating risedroante

and deoxy-risedronate by HPLC on an Inertsil Phenyl-3 HPLC column by gradient elution with a mixture of solution A and solution B, wherein the amount of solution A is increased from about 10% (v/v) to about 95% (v/v), and wherein the solution A is about 90% (v/v) methanol in water, and solution B is about 10 mM ammonium acetate-acetic acid buffer with 2% (v/v) triethylamine.

Another particular embodiment of the invention is the method for quantitatively determining risedronate in a mouse or rat urine sample, comprising:

a) adding about 100 μL of about 10 μg/mL dexoy-risedronate solution in water to about 0.4 mL of the mouse or rat urine sample;
b) applying the mouse or rat urine sample to a 30 mg Oasis HLB cartridge, wherein the cartridge has been pre-conditioned with about 1 mL of methanol and followed by about 0.5 mL of 1% (v/v) TEA in water;
c) washing the cartridge with about 0.5 mL of 1% (v/v) TEA in water and about 0.5 mL of 1% (v/v) formic acid in methanol;
d) eluting risedronate with about 1.5 mL of a mixture of about 60% (v/v) methanol and about 40% (v/v) water containing about 3 mM EDTA under vacuum;
e) evaporating the eluted solution and reconstituting with about 100 μL of a mixture of about 10% (v/v) of methanol and about 90% (v/v) of about 0.05 M NH_4Ac-NH_4OH buffer to provide a sample mixture of risedronate and dexoy-risedronate; and
f) analyzing the sample mixture with a LC-MS/MS system, wherein risedronate and dexoy-risedronate are separated by HPLC on an Inertsil Phenyl-3 HPLC column by gradient elution with a mixture of solution A and solution B, wherein the amount of solution A is increased from about 10% (v/v) to about 95% (v/v), and wherein the solution A is about 90% (v/v) methanol in water, and solution B is about 10 mM ammonium acetate-acetic acid buffer with 2% (v/v) triethylamine.

Another particular embodiment of the invention is the method for quantitatively determining risedronate in a mouse or rat urine sample, comprising:

a) adding about 100 μL of about 10 μg/mL dexoy-risedronate solution in water to about 0.4 mL of the mouse or rat urine sample;
b) applying the mouse or rat urine sample to a 30 mg Oasis HLB cartridge, wherein the cartridge has been pre-conditioned with about 1 mL of methanol and followed by about 0.5 mL of 1% (v/v) TEA in water;

c) washing the cartridge with about 0.5 mL of 1% (v/v) TEA in water and about 0.5 mL of 1% (v/v) formic acid in methanol;

d) eluting risedronate twice with about 0.75 mL of a mixture of about 60% (v/v) methanol and about 40% (v/v) water containing about 3 mM EDTA under vacuum;

e) evaporating the eluted solution and reconstituting with about 100 μL of a mixture of about 10% (v/v) of methanol and about 90% (v/v) of about 0.05 M NH_4Ac-NH_4OH buffer to provide a sample mixture of risedronate and dexoy-risedronate; and

f) analyzing the sample mixture with a LC-MS/MS system, wherein risedronate and dexoy-risedronate are separated by HPLC on an Inertsil Phenyl-3 HPLC column by gradient elution with a mixture of solution A and solution B, wherein the amount of solution A is increased from about 10% (v/v) to about 95% (v/v), and wherein the solution A is about 90% (v/v) methanol in water, and solution B is about 10 mM ammonium acetate-acetic acid buffer with 2% (v/v) triethylamine.

It is to be understood that this invention covers all appropriate combinations of the particular embodiments referred thereto.

The present invention will appear more clearly from the following example that is presented as an illustration only and is not to be considered as limiting the invention in its scope.

EXAMPLE

Step 1: Preparation of Risedronate Urine Standards in Mouse or Rat Urine

A 1 mg/mL risedronate stock solution is prepared by dissolving risedronate in HPCL grade water. 250 μg/mL, 100 μg/mL and 50 μg/mL risedronate standard solutions are prepared by diluting 250 μL, 100 μL and 50 μL of the 1 mg/mL risedronate stock solution to 1 mL with HPLC grade water, respectively. Appropriate dilutions of 250 μg/mL, 100 μg/mL and 50 μg/mL risedronate standard solutions are performed to yield risedronate working solutions of 25 μg/mL, 10 μg/mL, 5 μg/mL, 2.5 μg/mL and 1 μg/mL. Riseronate urine standards, ranging from 10 ng/mL to 2500 ng/mL, are prepared by adding 4 μL of each working solution into 0.4 mL of mouse or rat control urine (i.e., urine from mouse or rat that is not dosed with risedronate).

Step 2: Sample Preparation

A 0.4 mL urine sample collected from mouse or rat that has been dosed with risedronate is transferred to a 10x75 mm glass culture tube. 100 μL of stock internal standard

solution, i.e., 10 μg/mL deoxy-risedronate solution in water, and 100 μL of 5% TEA in water are added to all the risedronate urine standards and the urine sample, respectively.

Sample extraction is performed with a 30 mg Oasis HLB cartridge (1 mL, manufactured and sold by Waters Corporation, Catalog No.: WAT094225). The cartridges are conditioned with 1 mL methanol and followed by 0.5 mL of 1% TEA in water. The urine sample is applied to the cartridge. The cartridge is washed with 0.5 mL of 1% TEA in water and 0.5 mL of 1% formic acid in methanol, and then the analyte and the internal standard are eluted using two consecutive elution steps with 0.75 mL of a mixture of 60% methanol in water containing 3 mM EDTA. During the samples extraction, a vacuum (5-15 Psi) is used at each step to pull the liquid through the cartridges. The eluted solution is dried under nitrogen at 37°C for 70 minutes and then the residue is reconstituted in 100 μL of 10% methanol/90% 0.05 M NH_4Ac-NH_4OH buffer (pH 9.26).

Step 3: LC-MS/MS analysis

The analysis of Risedronate is performed using API 4000 LC-MS/MS system (sold by MDS Sciex), including a Shimadzu LC-10AD pump, SCL-10A VP system controller, Leap Technologies HTS PAL autosampler and API 4000 triple quadrupole mass-spectrometer.

Risedronate and the internal standard are separated on an Inertsil Phenyl-3 HPLC column (3 μL, 50x 030 mm, manufactured and sold by Varian, Inc., Catalog No.: 0408-050x030) by gradient elution with a mixture of solution A and solution B in 6 minutes, wherein the amount of solution A is increased from 10% to 95%. The solution A is 90% methanol in water, and the solution B is 10 mM ammonium acetate-acetic acid buffer (pH=5.0) with 2% triethylamine. The flow rate is kept constant at 0.2 mL/min. The sample (10 μL reconstitute) is injected onto the HPLC column directly. The mass spectrometer is programmed to monitor the transition m/z 281.9→63.1 for risedronate under negative mode with a collision energy of –58 volts. The m/z 265.8→79.2 is used to measure deoxy-Risedronate internal standard. The source temperature is set at 350°C. An analytical run time of 6 minutes is employed.

Results

Linearity

The assay for risedronate mouse or rat urine standard is linear from 10 to 2500 ng/mL with an LLOQ of 10 ng/mL. The determined accuracy at LLOQ is 14.8% with a precision of 17.8% (n=3). The regression parameters obtained during the validation are reported in table 1.

Correlation coefficients of 0.98 or greater for risedronate are obtained from the day-to-day analysis. A typical calibration curve is represented in FIGURE 6.

Table 1. Regression parameters for risedronate in mouse urine

Curve No/Date	Regression Parameters		
	Slope (response/concentration)	Intercept (response at concentration 0)	Correlation Coefficient
Within-day			
#1	0.000260	0.00208	0.9993
#2	0.000257	0.00151	0.9961
#3	0.000232	0.00300	0.9895
Mean	0.000250	0.00220	0.9950
SD	0.000015		
CV%	6.2		
Day-to-day			
Day 1	0.000267	0.00331	0.997
Day 2	0.000229	0.000594	0.9974
Day 3	0.000260	0.00208	0.9993
Mean	0.000252	0.00199	0.9979
SD	0.000020		
CV%	8.0		

Specificity

A small peak, at retention time of 2.27, is found in mouse control urine, but the peak height of LLOQ (10 ng/mL) is more than three times of the intensity of the peak in the mouse control urine (see FIGURE 3 and FIGURE 4). Thus, risedronate concentration at 10 ng/mL can be quantified.

Precision

Precision is expressed as the percent coefficient of variation of the concentrations measured at each control level over the duration of the study. The within-day coefficient of variation for the controls in mouse urine ranged from 2.2 to 9.6%. The day-to-day precision

for the controls at 3 concentration levels ranged from 5.2 to 10.3 %. The within-day and the day-to-day precisions for the standards ranged from 0.3 to 26%.

Accuracy

The assay accuracy is expressed as the percent difference between the mean determined quality control concentration and the theoretical value.

The within-day assay accuracy for the mouse urine controls ranged from –5.3 to 4.4 %. The day-to-day accuracy is determined over three analysis days using the controls at 3 concentration levels. The day-to-day accuracy ranged from –4.0 to 6.0%. The accuracy from the back-calculated concentrations of the mouse urine standards is within ±15%.

Table 2. The accuracy and precision of mouse urine calibration standards of risedronate

Replicate	Nominal Concentration (ng/mL)							
Or date	10	25	50	100	250	500	1000	2500
Within-day								
#1	7.93	20.7	49.4	100	237	422	995	2460
#2	13.3	22	42.8	91.6	238	388	868	2470
#3	9.94	25.4	50.6	97	253	476	976	2500
Mean	10.39	22.7	47.6	96.2	243	429	946	2477
SD	2.71	2.43	4.20	4.26	8.96	44.38	68.50	20.82
CV%	26.1	10.7	8.8	4.4	3.7	10.4	7.2	0.8
Diff%	3.9	-9.2	-4.8	-3.8	-2.9	-14.3	-5.4	-0.9
Day-to-day								
Day 1	13.8	24.7	46.9	93.5	237	532	1120	2610
Day 2	10.7	21.5	50.6	107	248	501	1010	2640
Day 3	9.94	25.4	50.6	97	253	476	976	2500
Mean	11.48	23.9	49.4	99.2	246	503	1035	2583
SD	2.04	2.08	2.14	7.01	8.19	28.05	75.27	73.71
CV%	17.8	8.7	4.3	7.1	3.3	5.6	7.3	2.9
Diff%	14.8	-4.5	-1.3	-0.8	-1.6	0.6	3.5	3.3

Table 3. The accuracy and precision of mouse urine quality controls of risedronate

Date	Nominal Concentration (ng/mL)		
	25	**250**	**2500**
Day 1	28.2	257	2900
	30	290	2580
	26.1	236	2870
Day 2	28.9	226	2650
	27.2	208	2800
	25.5	238	2470
Day 3	27.1	244	2650
	24.1	246	2670
	24.4	231	2520
	23.8	204	2600
	20.6	259	2610
Within-day (Day 3)			
Mean	24.0	237	2610
SD	2.31	20.8	57.9
CV %	9.6	8.8	2.2
Diff %	-4	-5.28	4.4
Day-to-day			
Mean	26.0	240	2665
SD	2.69	24.1	138
CV%	10.3	10.1	5.2
Diff%	4.0	-4.0	6.6

Recovery

A recovery of risedronate in extracted mouse urine as compared to extracted mouse urine blank with neat spiked is around 51.3%

Table 4. Recovery of risedronate from mouse urine samples (250 ng/mL)

Replicates	Extracted Urine Sample (peak area)	Extracted Blank Urine Sample + spiked neat peak area
#1	12400	30400
#2	18000	28800
#3	16600	29800
#4	18100	36000
#5	14500	30300
Mean	15920	31060
SD	2447	2833
CV%	15.4	9.1
Recovery (%)	51.3	

The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof.

We claim:

1. A method for quantitatively determining risedronate in a urine sample comprising:
   a) adding an internal standard solution to the urine sample;
   b) applying the urine sample to an Oasis HLB cartridge, wherein the cartridge has been pre-conditioned with methanol;
   c) washing the cartridge with about 1% (v/v) TEA in water and about 1% (v/v) formic acid in methanol;
   d) eluting risedronate, at least once, with a mixture of about 60% (v/v) methanol and about 40% (v/v) water containing about 3 mM EDTA under vacuum;
   e) evaporating the eluted solution and reconstituting with a mixture of 10% (v/v) methanol and 90% (v/v) 0.05 M NH_4Ac-NH_4OH buffer to provide a sample mixture of risedronate and the internal standard; and
   f) analyzing the sample mixture with a LC-MS/MS system.
2. The method according to claim 1, wherein the urine sample is a mouse or rat urine sample.
3. The method according to claim 1, wherein the internal standard is deoxy-risedronate.
4. The method according to claim 1, wherein the internal standard is deoxy-risedronate that is added as a solution in water.
5. The method according to claim 1, wherein the internal standard is deoxy-risedronate that is added as an about 10 μg/mL solution in water.
6. The method according to claim 1, wherein the Oasis HLB cartridge is a 30 mg Oasis HLB cartridge.
7. The method according to claim 1, wherein the amount of the urine sample is about 0.4 mL.
8. The method according to claim 1, wherein the cartridge has been pre-conditioned with about 1 mL of methanol.
9. The method according to claim 1, wherein the cartridge has been pre-conditioned with about 1 mL of methanol and followed by about 0.5 mL of 1% (v/v) TEA in water.
10. The method according to claim 1, wherein the cartridge is washed with about 0.5 mL of about 1% (v/v) TEA in water and about 0.5 mL of about 1% (v/v) formic acid in methanol.
11. The method according to claim 1, wherein the amount of the mixture of about 60% (v/v) methanol and about 40% (v/v) water containing about 3 mM EDTA is about 1.5 mL.

12. The method according to claim 1, wherein risedronate is eluted twice with about 0.75 mL of the mixture of about 60% (v/v) methanol and about 40% (v/v) water containing about 3 mM EDTA.

13. The method according to claim 1, wherein the amount of the mixture of 10% (v/v) methanol and 90% (v/v) 0.05 M NH_4Ac-NH_4OH buffer is about 100 μL.

14. The method according to claim 1, wherein the internal standard is deoxy-risedronate, and analyzing the sample mixture with a LC-MS/MS system comprisese separating risedroante and deoxy-risedronate by HPLC on an Inertsil Phenyl-3 HPLC column by gradient elution with a mixture of solution A and solution B, wherein the amount of solution A is increased from about 10% (v/v) to about 95% (v/v), and wherein the solution A is about 90% (v/v) methanol in water, and solution B is about 10 mM ammonium acetate-acetic acid buffer with 2% (v/v) triethylamine.

15. A method for quantitatively determining risedronate in a mouse or rat urine sample, comprising:

a) adding about 100 μL of about 10 μg/mL dexoy-risedronate solution in water to about 0.4 mL of the mouse or rat urine sample;

b) applying the mouse or rat urine sample to a 30 mg Oasis HLB cartridge, wherein the cartridge has been pre-conditioned with about 1 mL of methanol and followed by about 0.5 mL of 1% (v/v) TEA in water;

c) washing the cartridge with about 0.5 mL of 1% (v/v) TEA in water and about 0.5 mL of 1% (v/v) formic acid in methanol;

d) eluting risedronate with about 1.5 mL of a mixture of about 60% (v/v) methanol and about 40% (v/v) water containing about 3 mM EDTA;

e) evaporating the eluted solution and reconstituting with about 100 μL of a mixture of about 10% (v/v) of methanol and about 90% (v/v) of about 0.05 M NH_4Ac-NH_4OH buffer to provide a sample mixture of risedronate and dexoy-risedronate; and

f) analyzing the sample mixture with a LC-MS/MS system, wherein risedronate and dexoy-risedronate are separated by HPLC on an Inertsil Phenyl-3 HPLC column by gradient elution with a mixture of solution A and solution B, wherein the amount of solution A is increased from about 10% (v/v) to about 95% (v/v), and wherein the solution A is about 90% (v/v) methanol in water, and solution B is about 10 mM ammonium acetate-acetic acid buffer with 2% (v/v) triethylamine.

16. A method for quantitatively determining risedronate in a mouse or rat urine sample, comprising:

a) adding about 100 μL of about 10 μg/mL dexoy-risedronate solution in water to about 0.4 mL of the mouse or rat urine sample;

b) applying the mouse or rat urine sample to a 30 mg Oasis HLB cartridge, wherein the cartridge has been pre-conditioned with about 1 mL of methanol and followed by about 0.5 mL of 1% (v/v) TEA in water;

c) washing the cartridge with about 0.5 mL of 1% (v/v) TEA in water and about 0.5 mL of 1% (v/v) formic acid in methanol;

d) eluting risedronate twice with about 0.75 mL of a mixture of about 60% (v/v) methanol and about 40% (v/v) water containing about 3 mM EDTA;

e) evaporating the eluted solution and reconstituting with about 100 μL of a mixture of about 10% (v/v) of methanol and about 90% (v/v) of about 0.05 M NH_4Ac-NH_4OH buffer to provide a sample mixture of risedronate and dexoy-risedronate; and

f) analyzing the sample mixture with a LC-MS/MS system, wherein risedronate and dexoy-risedronate are separated by HPLC on an Inertsil Phenyl-3 HPLC column by gradient elution with a mixture of solution A and solution B, wherein the amount of solution A is increased from about 10% (v/v) to about 95% (v/v), and wherein the solution A is about 90% (v/v) methanol in water, and solution B is about 10 mM ammonium acetate-acetic acid buffer with 2% (v/v) triethylamine.

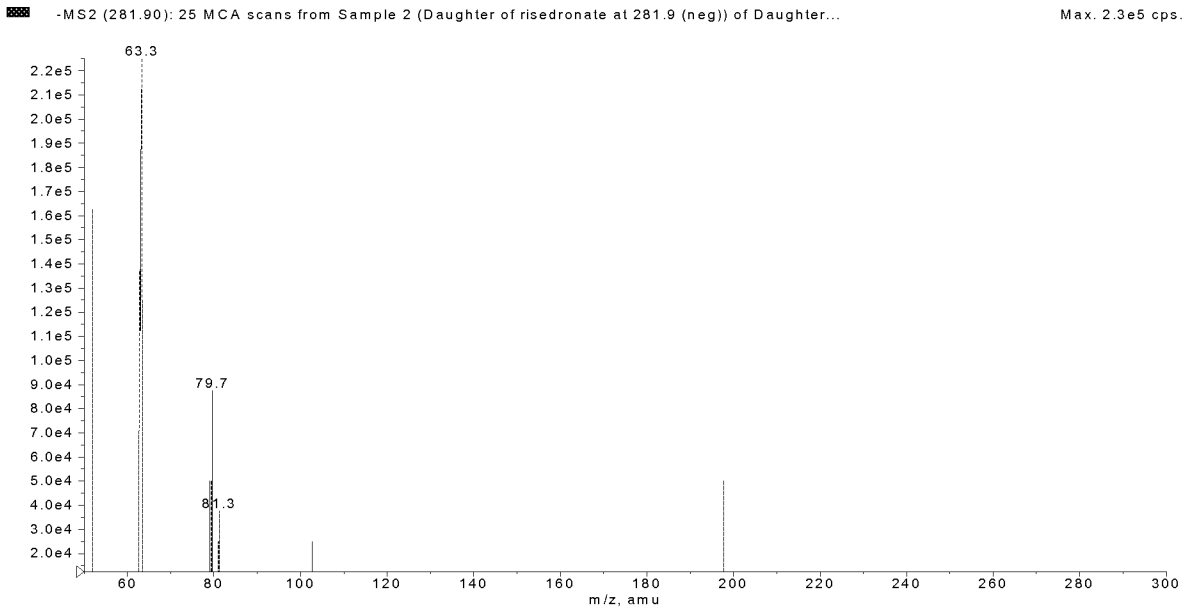


FIGURE 1. ESI-MS Spectrum of Risedronate

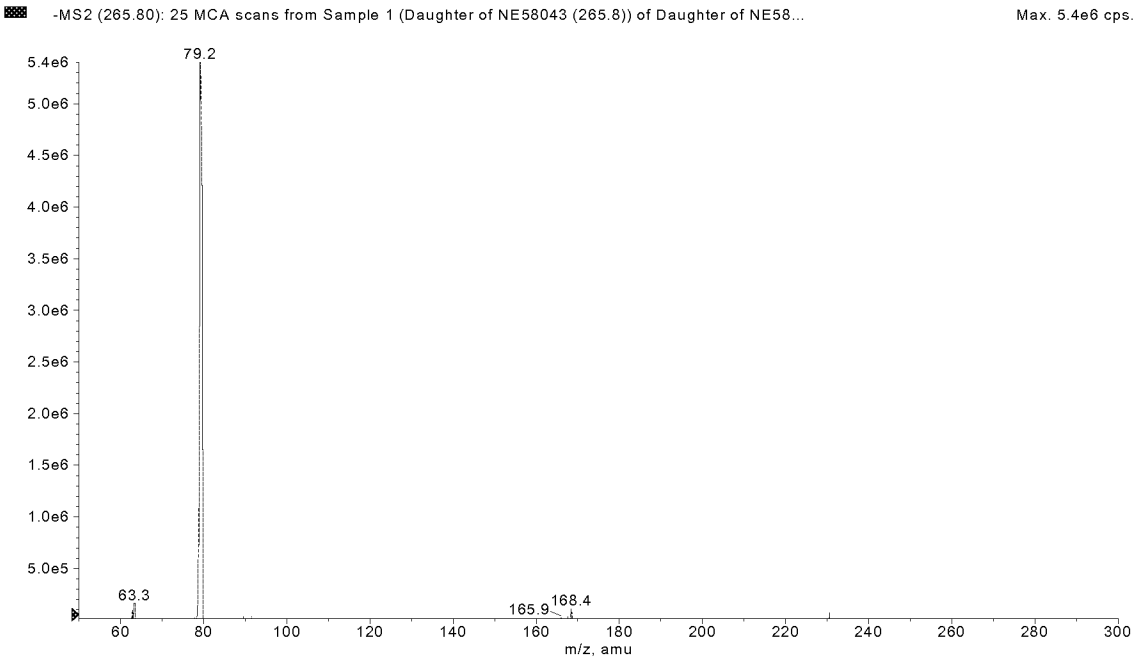


FIGURE 2. ESI-MS Spectrum of Deoxy-Risedronate

FIGURE 3. Chromatogram of Risedronate in Mouse Urine (LLOQ 10 ng/mL)

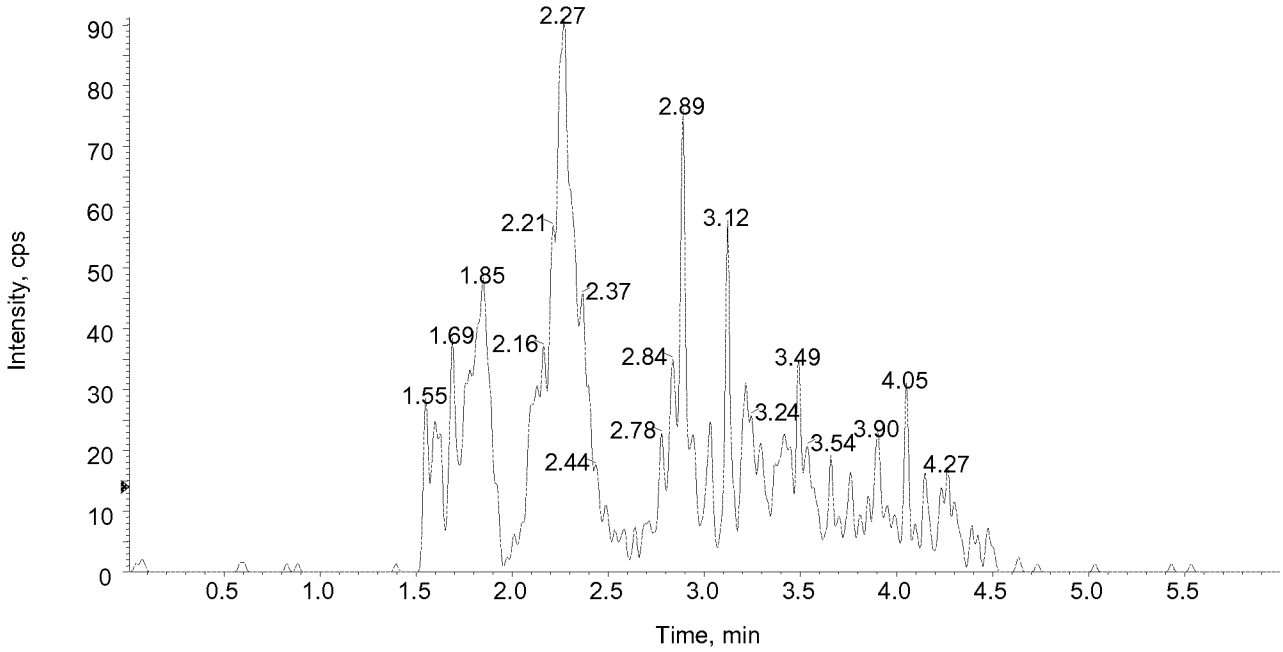


FIGURE 4. Chromatogram of Mouse Control Urine

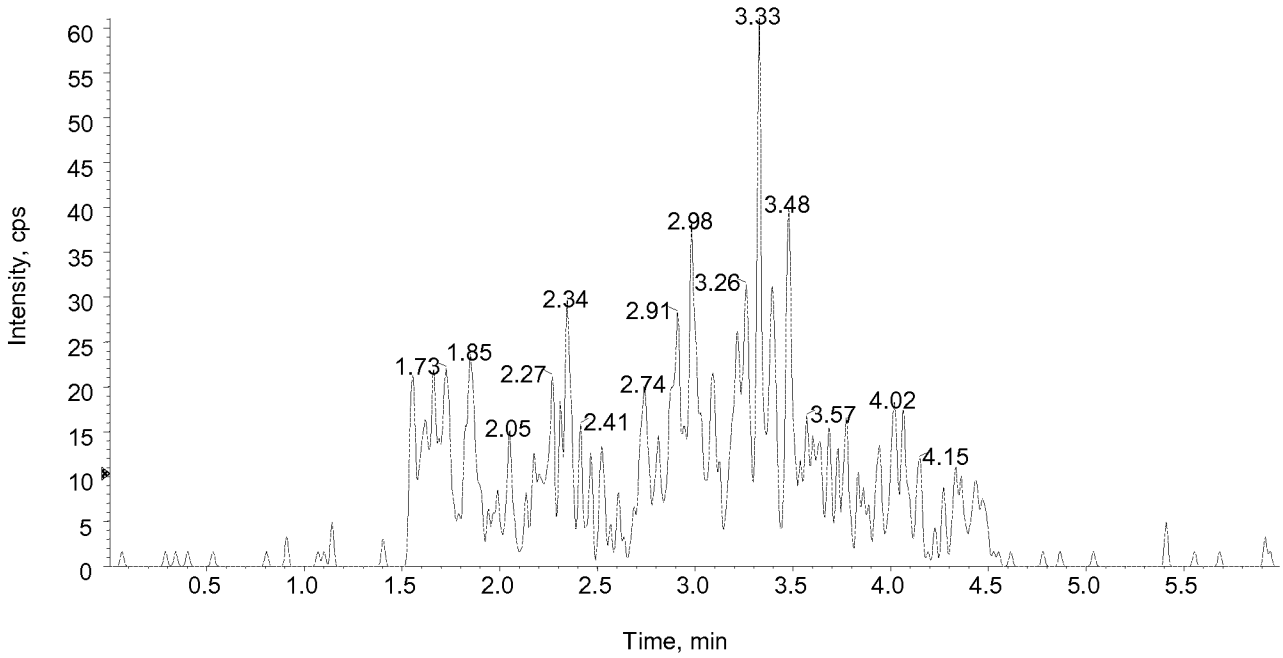


FIGURE 5. The Typical Chromatogram of Risedronate and Deoxy-Risedronate

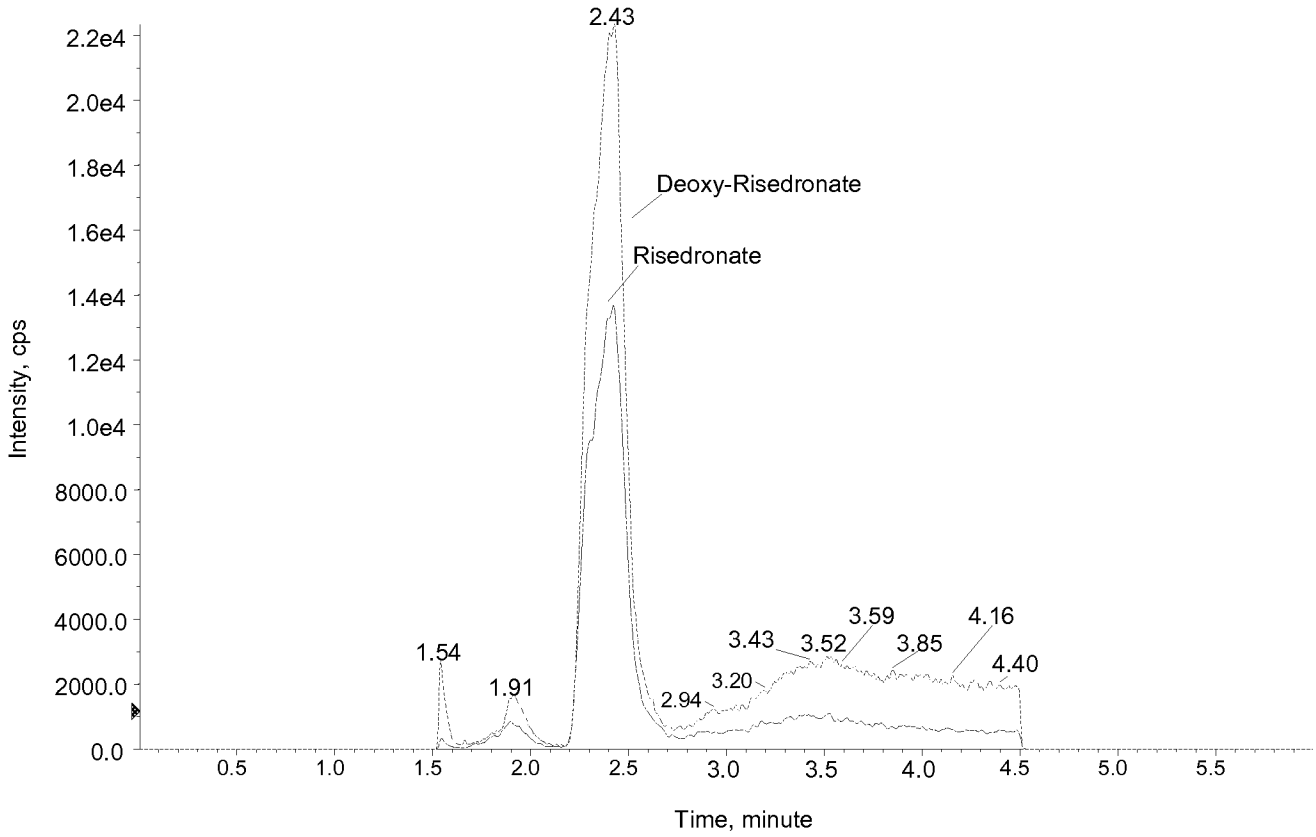
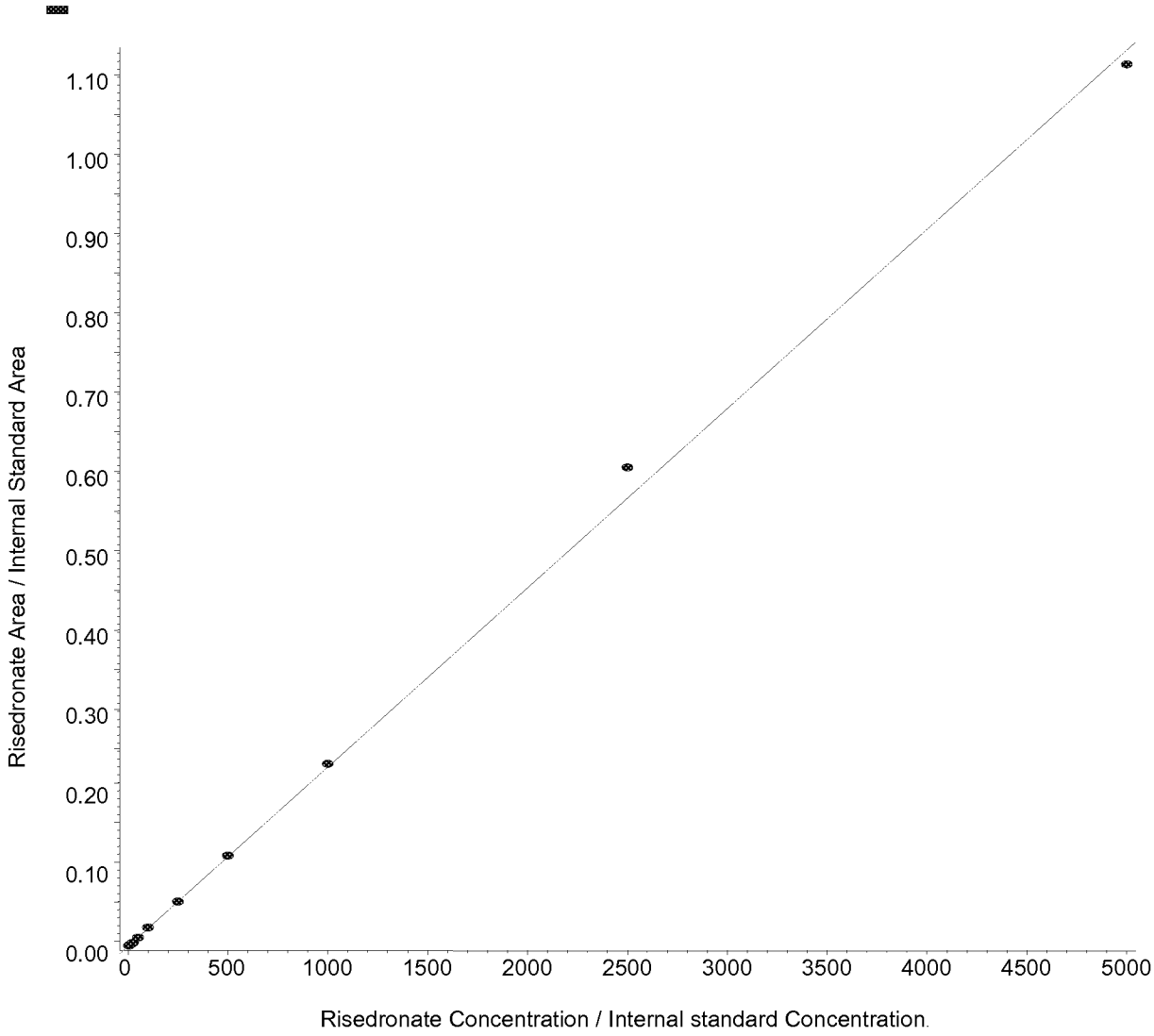


FIGURE 6. The Typical Calibration Curve of Risedronate in Mouse Urine



INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/055849

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/52 G01N30/00 B01D15/36

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N B01D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2006/127973 A (WATERS INVERSTMENTS LTD [US]; LU ZILING [US]; DIEHL DIANE [US]; MAZZEO) 30 November 2006 (2006-11-30) the whole document in particular: paragraphs [0016], [0017], [0037], [0041], [0044], [0045] examples 1-3 --- -/--	1-16

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 May 2008	09/06/2008

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Tuynman, Antonin

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/055849

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	"OASIS TM Sample Extraction Products" WATERS OASIS SAMPLE EXTRACTION PRODUCTS, [Online] 1999, pages 1-10, XP002481384 Retrieved from the Internet: URL:HTTP://WWW.WOONGKI.COM/PRODUCT/SAMPLE_HANDLING/EXTRACTIONS/HLB.PDF> [retrieved on 2008-05-23] the whole document in particular: figure 10 page 5, line 24 - line 48	1-16
A	ZHU LEE S ET AL: "A general approach for the quantitative analysis of bisphosphonates in human serum and urine by high-performance liquid chromatography/tandem mass spectrometry" RAPID COMMUNICATIONS IN MASS SPECTROMETRY, vol. 20, no. 22, 2006, pages 3421-3426, XP002481339 ISSN: 0951-4198 cited in the application the whole document	1-16
A	TURCOTTE S ET AL: AAPS PHARMSCI, vol. 5, no. 4, 2003, page M1357, XP002481361 cited in the application abstract	1-16
A	VALLANO P T ET AL: "Determination of risedronate in human urine by column-switching ion-pair high-performance liquid chromatography with ultraviolet detection" JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL SCIENCES & APPLICATIONS, ELSEVIER, AMSTERDAM, NL, vol. 794, no. 1, 25 August 2003 (2003-08-25), pages 23-33, XP004441225 ISSN: 1570-0232 the whole document	1-16

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2008/055849

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2006127973 A	30-11-2006	NONE	