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LC–APCI-MS–MS Method for the Tissue Distribution of Viaminate After Oral Administrations to Rats

Ling Cao[1,2], Feng Zheng[1], Pengcheng Ma[3,*], Wenying Liu[1,*], Di Sun[1], Xuan Chen[1], Yanwei Lai[1], and **Meng Gou[1]**

[1]Department of Pharmaceutical Analysis, China Pharmaceutical University, 210009; [2]Jiangsu Institute for Drug Control, 210008, and [3]Institute of Dermatology, CAMS and PUMC, 210042, Nanjing, China

Abstract

Fast and sensitive liquid chromatography–atmospheric pressure chemical ionization-mass spectrometry (LC–APCI-MS) method for the specific determination of viaminate in 14 kinds of rat tissues pre-treated with simple procedure was developed and validated. Biological samples were prepared by direct precipitation to skin, stomach, intestine, and liver and extracted by liquid–liquid extraction to lung, kidney, muscle, spleen, brain, fat, testes (male and female), eye, and heart. After addition of menaquinon as internal standard to tissue homogenate, the supernatant was injected into the isocratic chromatographic system using a Waters Symmetry C8 column and methanol–water–formic acid (93:7:0.1) as the eluent. The eluate was completely led into an APCI interface with selected ion monitoring mode and the analytes were quantified using triple quadrupole MS. The assays were successfully validated in the ranges 0.02~20 ng/mL for lung, 0.02~10 ng/mL for kidney, spleen, muscle, brain, fat, eye, and heart, 0.05~10 ng/mL for testicle, 0.4~100 ng/mL for liver, skin and intestine, and 1.0~200 ng/mL for stomach. The accurate and precise studies showed good reproducibility with coefficients of variation below 8.5% and the recoveries range from 90 to 109%. The analytes were chemically stable under all relevant conditions and the assays were applied in tissue distribution study. The results showed that the viaminate concentration was high in skin, low in kidney, and almost undetectable in eye and brain.

Introduction

Viaminate is a derivative of retinoid, which was prepared by all-trans retinoid acid and *p*-amino-benzoic acid ethyl ester. It has effective clinical evaluation for diminishing inflammation and treating acne by adjusting and controlling the differentiation and development of epithelia, reducing the secretion of sebum and restraining the growth of Propionibacteria of acne (1–2) for nearly 30 years. In clinic, it is approbatory that viaminate possesses the therapeutic effects of retionid but with less toxic side effects.

Although viaminate has been used in clinic for nearly three decades, specific and sensitive method suitable for its determination in plasma and tissues are very limited. Only one reference using high-performance liquid chromatography (HPLC) method to study the pharmacokinetic characteristics of viaminate in rat plasma in 1992 by Tang Xin et al. was reported (3). But the lower limit of quantification (LLOQ) of this method was 100 ng/mL. And the approach of administering the sample was by injected into the vein, which was different to the clinical use. Recently, Jin Ying et al. reported the study on the pharmacokinetic of viaminate in human plasma by LC–ESI(–)-MS–MS (4). But there are scarcely any reports for methods to analyze viaminate in rat tissue samples and no reports of the tissue distribution study in rat. It is essential to establish a precise and sensitive method to determine the lower concentration of viaminate and apply the method to the rat tissues distribution study.

The isomerization to light is the most important challenge attribute to its double bonds, therefore the whole operation must be carried out in dark room lighting with only a low-intensity red light source. The whole operation processing steps especially dissection and sampling is very inconvenient.

The liquid–liquid extraction was required basing on the low concentration in part tissues. But viaminate was adsorbed on the test vessels very strongly. The glass vessels, such as a centrifugal tube, must be washed with pure methanol to remove the leftover. Also, it is necessary to wash the injection needle of the HPLC with a congruent solution.

The extremely low concentration of viaminate in rat part tissue, such as eye and brain, was another challenge. The lowest

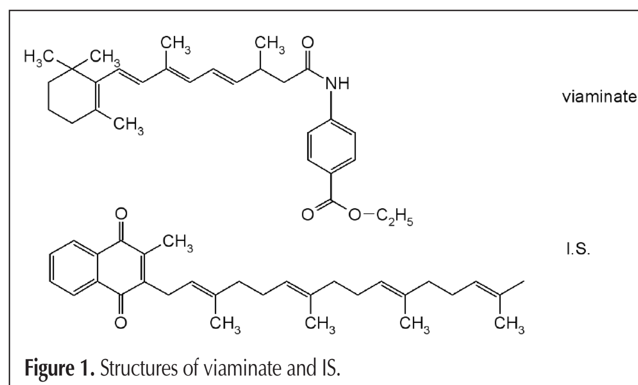

Figure 1. Structures of viaminate and IS.

* Authors to whom correspondence should be addressed: email mpc815@163.com (Peng-Cheng Ma), lwcpu@126.com (Wenying Liu).

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content was no more than 0.1 ng/mL. So the method using HPLC–UV or LC–MS-Trap detection was unsuitable for the detection of viaminate in rat tissues. The LC–ESI(–)-MS detection was also not sensitive enough to detect the concentration of viaminate, which its LLOQ was 0.1 ng/mL (4). Therefore, it is an important prerequisite to develop a highly sensitive detection method for the pharmacokinetic study of viaminate first of all.

Since 1995, HPLC–mass spectrometric-based methods incorporating atmospheric pressure chemical ionization (APCI) coupled with quadrupole mass analyzers have been used increasingly for carotenoids and other analyte determinations in sample (5). It is a very sensitive technique for the ionization of lipids, which can provide efficient ion production for MS detection, and has been used for the analysis of retinoid acid (6–7). In this paper, a high sensitive and reproducible method for the determination of viaminate in rat tissues, using LC–APCI-MS–MS was developed and can resolve these problems above.

Experimental

Materials

Viaminate standard (purity ≥ 98%) was kindly provided by Chongqing Huabang pharmacy company Ltd. (Chongqing, Sichuan, China). Menaquinon as internal standard (IS) was donated by Department of Pharmaceutical Analysis, China Pharmaceutical University (Nanjing, Jiangsu, China). The structures of viaminate and IS are shown in Figure 1. HPLC-grade methanol, acetonitrile, and formic acid were obtained from Tedia Company, Inc. (Fairfield, OH). All other reagents and solvents were all of analytical grade. Purified water (Milli-Q Biocel Ultrapure Water system, Millipore, MA) was used for all aqueous solutions.

Sprague-Dawley (SD) (male or female, 200 ± 25 g) rats were supplied by the Laboratory Animal Center of Nanjing Medical University (Nanjing, Jiangsu, China). The blank tissue for the preparation of methodology was obtained from them (drug-free).

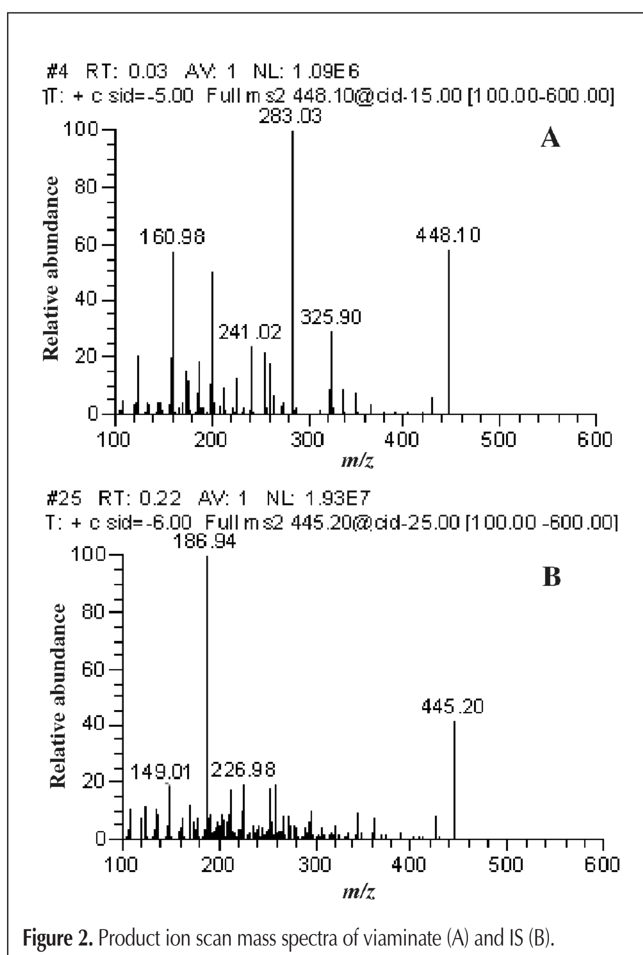

Figure 2. Product ion scan mass spectra of viaminate (A) and IS (B).

HPLC conditions

The liquid chromatograph was performed on a Finnigan surveyor HPLC system (Thermo Electron Corp., San Jose CA) with conditioned autosampler at 4°C equipped with a 25 µL injection loop. The separation was carried out on a Waters Symmtry C8 column (150 mm × 4.6 mm i.d., 5-µm particle size, Waters Corp., Milford, MA) equipped with a coupled to a guard column (10 mm × 4.6 mm i.d., 5 µm particle size). The column temperature was set at 25°C with column oven. The analysis was achieved with methanol–water–formic acid (93:7:0.1)(v/v/v) as the mobile phase at a flow rate of 1.0 mL/min. The injection volume was 20 µL using partial loop mode for sample injection.

MS

Mass spectrometric detection was carried out on a Thermo Finnigan Surveyor LC-TSQ Quantum Ultra AM tripe-quadrupole tandem MS (Thermo Electron Corp.) with an APCI interface. The APCI source was set in positive ionization mode. System control and data acquisition was performed with Xcalibur 1.1 data software. Peak integration and calibration were carried out using LC Quan software (Thermo Electron Corp.). Quantitation was performed using selected ion monitoring (SRM) scan mode of the transitions of *m/z* 448 → 283 for viaminate and *m/z* 445 → 187 for menaquinon (I.S.), respectively (Figure 2). Both Q1 and Q3 peak widths were set at 0.7 Th. The optimal MS parameters were as follows: discharge current 10.0 A, vaporizer temperature 420°C, capillary temperature 330°C. The high purity nitrogen sheath gas and the auxiliary gas pressure were set at 35 and 5 psi, respectively. The heated argon was used as the collision-induced dissociation (CID) gas at a pressure of 1.20 mTorr. The collision energy in the in-source CID mode was set at 6 eV, and the collision energy in the MS–MS mode was set at 15 eV and 25 eV for viaminate and IS, respectively.

The entire effluent volume from HPLC was directed to the APCI(+)–MS. After each sample run, the autosampler was flushed with pure methanol to remove strongly retained residues in needle. To prevent interfering material in the HPLC eluate from entering the APCI source, a divert valve was utilized and the eluate flow destination was switched to waste in the first 2.5 min of the chromatographic run and afterwards to the ion source.

Preparation of standard solutions

Stock solution of viaminate was prepared in methanol at 2 µg/mL and was stored at 4°C and used within 1 month of preparation. Working solutions were obtained by serial dilution of stock solution with methanol. Dilutions were performed imme-

diately prior to use. The calibration standards for viaminate were prepared by spiking blank tissue homogenates at 0.4, 1, 4, 10, 20, 40, 100, 200 ng/mL for method A and 0.02, 0.05, 0.1, 0.2, 0.5, 2, 5, 10 ng/mL for method B. The quality control (QC) samples of viaminate were similarly prepared at 1, 10, and 100 ng/mL for method A, and 0.05, 0.5, and 5 ng/mL for method B. The IS stock solution was prepared in methanol containing 5 µg/mL. The stock solution was diluted with acetonitrile to obtain internal standard solution A containing 50 ng/mL and with ethanol to obtain internal standard solution B containing 5 ng/mL, respectively, and both the internal standard solutions were used as the precipitator of protein as well.

Sample preparation

All rat tissue homogenates were prepared by adding purified water to these tissues at a ratio of 5:1 (mL:g) and then homogenized.

Method of precipitation (method A)

The following method was suitable to prepare the sample of the tissue of skin, stomach, intestine, and liver. To 200 µL of homogenate samples in 2 mL centrifugal tube, 800 µL of the internal standard solution A was added. The mixture was vortexed for 3 min. After centrifugation at 15000 r.p.m. for 10 min, the clear supernatant was transferred to a brown autosampler vial and injected into the LC–MS system for analysis.

Method of extraction (method B)

To prepare the sample of the tissue of lung, kidney, muscle, spleen, brain, fat, testes, eye, and heart, the following method was used. One milliliter of the internal standard solution B was added to 500 µL of homogenate sample in 10 mL centrifugal tube. The mixture was vortexed for 1 min, to which 3 mL of cyclohexane was added, and then vortexed again for 3 min. After centrifugation for 5 min at 4000 rpm, the cyclohexane layer was transferred to another 10 mL tube and dried under a stream of nitrogen (37°C). The residue was dissolved in 200 µL methanol. After centrifugation at 15,000 rpm for 10 min, the clear supernatant was used for analysis.

The samples with concentrations greater than the maximum standard in the calibration curve were quantitatively diluted with purified water.

All previously mentioned processing steps must be performed in a room with only a low-intensity red light source to minimize its photoisomerization.

Method validation

The method was validated in all above tissues. The specificity of the assay for viaminate in the presence of endogenous components of rat tissues was investigated by comparing chromatograms of blank tissues with those of corresponding standard tissue sample spiked with viaminate and IS (5 ng/mL) and tissue sample after oral administration of viaminate in rat.

Calibration curves based on seven or eight spiked samples as described earlier were prepared to validate the linearity of the method for different tissues. Each calibration curve was constructed by weighted linear regression (1/x2) of analyte-IS peak area ratio. To determine the precision of the method, three QC samples of all tissues at three concentration levels (low, mid, and high levels of the calibration range) were analyzed three times on the same day. Mean and relative standard deviation was calculated. The accuracy of the method was evaluated by analyzing recovery percentages. Recoveries were calculated by using the ratio of the detected to the added.

The LLOQ was defined as the lowest concentration of the analysts quantified at which the signal-to-background noise ratio was greater than 10:1.

Application

To minimize photoisomerization of viaminate, the acquisition of all subsequent processing steps were carried out in a room protected from direct sunlight and lit by only a low-intensity red light source.

The analytical method described above was subsequently applied to quantify samples for rat tissues distribution studies. 30 Sprague-Dawley rats, including of 15 male and 15 female, were chosen and randomly grouped into five pools, each of which consisted of 3 male and 3 female. These SD rats were fasted 12 h before drug sampling.

Viaminate was minced and dispersed by 0.1% sodium carboxymethylcellulose to 0.5 mg/mL. Then 5 mg per kg of viaminate was administered to the rats via gastric gavage. Six rats were sacrificed at the time points of 1, 3, 8, 12, 24 h after administered, respectively. Tissue samples were immediately collected from the lung, kidney, muscle, spleen, brain, fat, testicle, eye, heart, skin, stomach, intestine, and liver of each rat and were put into the physiological saline to exclude the remaining bloodstain. They were dried the moisture with filter paper and weighed. About 0.5 g of each tissue was taken and then 2.5 mL of each homogenate (20%, w/v) was prepared as analytical sample.

Results and Discussion

Method development

Our previous study showed that the sensitivity of viaminate detecting by APCI(+)-MS–MS was 10 times higher than ESI(–)-MS–MS (4). The lower limit of quantification (LLOQ) of viaminate by APCI(+)-MS–MS was less than 0.05 ng/g (0.01 ng/mL). This high sensitivity has obvious advantage to determine the small quantity of viaminate in rat tissues.

As a derivative of retinol, viaminate is a lipophilic compound. Several sample treatment methods have been reported for retinol in plasma and tissues (8–14). Our preliminary experiments showed that the concentration of viaminate in stomach, intestine, skin, and liver was high enough to adopt a simple protein precipitate method using acetonitrile to prepare sample. Because of the low concentration of viaminate in lung, kidney, muscle, spleen, brain, fat, testes, eye and heart of rat, a quite complex sample treating procedure including protein precipitation, liquid–liquid extraction, drying by nitrogen and re-dissolving sample must be used before analysis. Although the procedure is complex and time-consuming, it is successfully

applied in rat tissues. Whichever methods were used, the analyte was efficiently extracted from rat tissues.

Due to the strong retention of viaminate, the C8 was selected as analytical column. Because it has the long conjugated double bonds structure, steady and high extraction recovery and appropriate retention, Menaquinon was chosen as internal standard of analysis of viaminate.

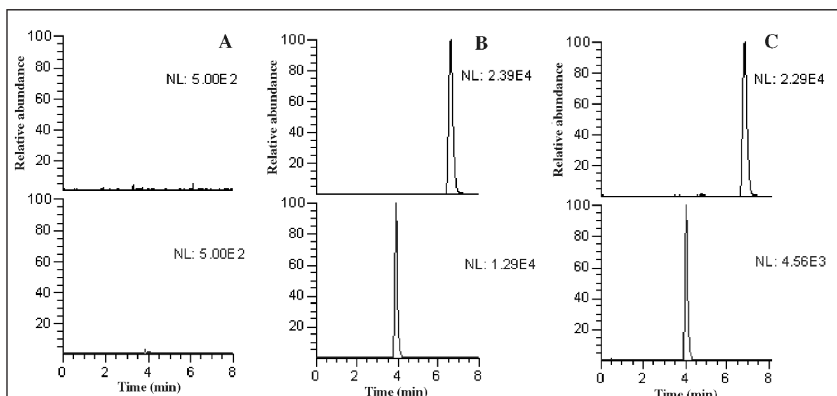

Figure 3. SRM chromatograms of blank skin homogenate (A), blank skin homogenate spiked with viaminate and IS (B), and skin sample from a subject 3 h after the administration of viaminate, in which the concentration of viaminate was found to be 151.2 ng/g (C).The retention time is 4.0 min for viaminate and 7.3 min for the IS.

The strong adsorption of viaminate to the LC–MS autosampler needle causes difficult analysis. Although the adsorbed needle was washed several times with the mixture of methanol–water (50:50) recommended by the LC–MS instrument manufacturer, the leftover still existed. Based on the experiments, absolute methanol was used instead to wash the needle, and the adsorption was largely eliminated.

Our study indicated that the matrix effect on viaminate by APCI was much lower than LC–ESI(–)-MS–MS. Table I shows the results of the liver.

Method validation

The method selectivity for all above tissues was investigated. The typical LC–SRM chromatograms obtained from blank skin tissue sample, corresponding standard skin tissue sample spiked with viaminate and IS and skin tissue sample after administration of viaminate in rat are shown in Figure 3 and all tissue samples after administration of viaminate in Figure 4. The retention time of viaminate and IS were 3.9 and 6.5 min, respectively. There were no

Figure 4. Chromatograms of rat tissue samples after administration of viaminate stomach (A), intestine (B), skin (C), liver (D), lung (E), kidney (F), muscle (G), spleen (H), brain (I), fat (J), testes (male) (K), testes (female) (L), eye (M), and heart (N). The retention time is 4 min for viaminate and 7 min for the IS.

significant interfering peaks at the retention times of viaminate and IS in all blank tissue samples.

Linear calibration curves were obtained in the given concentration range of viaminate in each tissue sample, respectively (Table II). The calibration curves for viaminate were linear in the range of each tissue homogenate. The correlation coefficient (r) were all greater than 0.99. The LLOQ (S/N > 10) of viaminate was 50 pg/g. The results of accuracy and precision are summarized in Table III. The data demonstrated that the method is accurate and precise with coefficients of variation below 8.5% for all the samples. The recoveries of viaminate range from 90 to 109% for tissue homogenates.

The samples for the recovery test stored at room temperature for 12 h did not suffer any appreciable changes in assay value. The mean values (n = 3) for accuracies were within 5.43% of their expected values, and percentage variation was less than 6.12% for all the samples. And the QC samples after three freeze (–20°C) and thaw (ambient) cycles are stable too. The mean (n = 3) observed values were within 4.89% of the expected values, and percentage variation was less than 6.21%.

Tissue distribution analysis

There was a very wide tissue distribution of viaminate in rats at 1 h after oral administration as shown in Table IV. Viaminate can be distributed to almost all rat tissues except eye and brain. The viaminate concentration reached a maximum at 3 h in most tissues except that at 8 h in liver. The concentration began to decline after 8 h and reduce to quite low at 24 h in almost all tissues. The observed distribution consequence in viaminate concentration was as follows: stomach > intestine > skin > lung > fat > testes (female) > muscle > liver > heart > testes (male) > kidney > spleen > eye > brain. The viaminate concentration in skin was the highest reached at 138.8 ng/g at 3 h, except for the stomach and intestine. Contrarily, the concentration in kidney was only 14.5 ng/g and was almost not detected in eye and brain. So viaminate can be used for the treatment of skin diseases and

Table I. Compared Matrix Effect Results of Viaminate in Liver by APCI with ESI (n = 3)

Concentration (ng/mL)	Recovery (%) (APCI)		Recovery (%) (ESI)	
	Mean ± SD	C.V.	Mean ± SD	C.V.
1	96.66 ± 0.66	0.68	72.84 ± 2.74	3.76
10	96.56 ± 1.32	1.37	77.19 ± 2.04	2.64
100	97.97 ± 1.57	1.60	79.20 ± 1.93	2.44

Table II. Calibration Curves for Viaminate in Rat Tissues

Sample matrix	Concentration (ng/mL)	Slope	Intercept (ng/mL)	Correlation coefficient (r)
Stomach	1.0~200	9.76E-4	–3.80E-4	0.9948
Intestine	0.4~100	9.90E-4	–1.72E-3	0.9987
Skin	0.4~100	1.92E-3	–5.30E-4	0.9931
Liver	0.4~100	9.48E-4	–9.00E-4	0.9958
Lung	0.02~20	1.44E-3	–1.21E-4	0.9957
Kidney	0.02~10	4.92E-3	–1.32E-3	0.9978
Muscle	0.02~10	1.31E-3	2.43E-4	0.9936
Spleen	0.02~10	8.84E-3	4.63E-4	0.9909
Brain	0.02~10	1.89E-2	1.85E-3	0.9977
Fat	0.02~10	1.27E-2	–3.80E-4	0.9928
Testis (male)	0.05~10	4.90E-3	–9.80E-5	0.9995
Testis (female)	0.05~10	5.38E-3	–1.70E-4	0.9909
Eye	0.02~10	4.40E-3	–6.68E-4	0.9929
Heart	0.02~10	7.84E-3	–9.35E-4	0.9944

Table III. Precision and Accuracy Data for Viaminate in Rat Tissues (n = 3)

Sample matrix	Concentration added (ng/mL)	Concentration found (ng/mL)	Recovery (%)	Precision (%)
Stomach	1.00	0.98	98.17	5.13
	10.00	10.33	103.29	2.85
	100.00	103.82	103.82	2.26
Intestine	1.00	1.00	99.51	6.95
	10.00	10.28	102.76	3.92
	100.00	94.20	94.20	1.98
Skin	1.00	0.93	93.31	7.47
	10.00	9.71	97.09	7.83
	100.00	90.53	90.53	4.50
Liver	1.00	1.01	101.08	4.80
	10.00	10.28	102.78	2.11
	100.00	102.51	102.51	2.05
Lung	0.050	0.053	105.23	8.42
	0.500	0.474	94.76	5.15
	5.000	5.036	100.72	3.69
Kidney	0.050	0.048	96.53	2.32
	0.500	0.473	94.59	5.52
	5.000	4.768	95.36	3.44
Muscle	0.050	0.053	106.47	6.40
	0.500	0.518	103.60	6.66
	5.000	4.969	99.38	3.39
Spleen	0.050	0.052	103.17	3.65
	0.500	0.501	100.14	4.12
	5.000	4.879	97.57	1.63
Brain	0.050	0.046	91.49	6.51
	0.500	0.514	102.87	7.32
	5.000	4.795	95.90	0.86
Fat	0.050	0.045	90.57	5.22
	0.500	0.500	100.04	5.74
	5.000	4.642	92.85	6.18
Testes (male)	0.050	0.047	94.71	7.71
	0.500	0.483	96.59	6.72
	5.000	5.235	104.69	2.38
Testes (female)	0.050	0.047	94.71	7.24
	0.500	0.524	104.87	4.78
	5.000	5.251	105.02	3.47
Eye	0.050	0.052	103.49	4.62
	0.500	0.544	108.80	1.84
	5.000	4.611	92.23	4.00
Heart	0.050	0.049	97.62	5.48
	0.500	0.472	94.40	5.17
	5.000	5.237	104.73	4.05

Table IV. Concentrations of Viaminate in Various Tissues of Rat at Times 1, 3, 8, 12, and 24 h After Single ig Dose of 5 mg/kg (*n* = 6)

	Concentration ± SD (ng/g)				
Tissue	1 h	3 h	8 h	12 h	24 h
Skin	23.4 ± 16.4	139 ± 44	18.0 ± 6.5	12.2 ± 1.4	4.11 ± 0.96
Liver	4.83 ± 1.00	12.9 ± 2.2	50.0 ± 22.1	5.14 ± 1.24	4.44 ± 1.36
Stomach	5225 ± 2357	32634 ± 9176	300 ± 100	70.1 ± 25.5	15.3 ± 5.4
Intestine	298 ± 100	1543 ± 611	59.5 ± 17.5	60.4 ± 13.9	8.94 ± 3.60
Lung	14.0 ± 4.5	122 ± 72	5.17 ± 2.09	1.81 ± 0.85	1.14 ± 0.57
Kidney	3.60 ± 3.01	14.5 ± 8.8	1.89 ± 0.55	2.02 ± 0.45	1.14 ± 0.32
Muscle	7.01 ± 3.08	52.1 ± 24.8	5.44 ± 3.42	6.49 ± 5.59	1.53 ± 0.81
Spleen	1.31 ± 0.55	12.8 ± 3.3	2.79 ± 1.53	1.52 ± 1.15	1.49 ± 0.94
Brain	0.07 ± 0.18	0.33 ± 0.33	0.03 ± 0.07	0.04 ± 0.06	0.16 ± 0.24
Fat	3.75 ± 1.93	89.3 ± 20.2	5.10 ± 1.83	3.82 ± 2.32	2.37 ± 1.39
Testis (male)	10.8 ± 5.3	58.3 ± 19.7	1.92 ± 0.12	1.57 ± 0.41	0.07 ± 0.07
Testis (female)	4.39 ± 1.84	17.0 ± 0.2	4.98 ± 1.35	1.10 ± 0.75	0.37 ± 0.18
Eye	0.25 ± 0.25	2.05 ± 1.86	0.31 ± 0.13	0.31 ± 0.26	0.48 ± 0.64
Heart	1.33 ± 0.46	49.0 ± 18.7	3.63 ± 2.09	1.34 ± 0.75	1.37 ± 0.72

the side effect on eye and kidney was little. These characteristics of viaminate are consistent with the clinical usage.

Conclusion

A validated and sensitive LC–APCI-MS–MS method for the determination of viaminate in rat tissues is described. This method represents an accurate and reproducible procedure for different tissue homogenates. The analyte was efficiently extracted from all rat tissue homogenates and no matrix effect can be observed. Using absolute methanol to wash needle of the autosampler, the strong adsorption of viaminate was greatly eliminated. The unique feature of these assays was the very high sensitivity, which the LLOQ reached 50 pg/g. It has been demonstrated to be usable in tissue distribution studies of viaminate. The tissue distribution results achieved may be useful for further study of the bioactive mechanism of viaminate.

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