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Short communication

Development and validation of a LC–MS/MS method for the determination of viaminate in human plasma

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Abstract

A sensitive and specific method for determination of viaminate in human plasma by using high-performance liquid chromatography coupled with electrospray tandem mass spectrometry (LC–MS/MS) was developed in this study. The plasma samples were simply deproteinated, extracted, evaporated, and then reconstituted in 200 μl of methanol prior to analysis. Chromatographic separation was carried out on a Shimadzu VP-ODS column (250 mm × 2.0 mm, 5 μm) with a mobile phase of methanol–water (95:5, v/v) at a flow rate of 0.2 ml/min. Quantification was performed in the negative-ion electrospray ionization mode by selected ion monitoring of the product ions at *m/z* 164 for viaminate and *m/z* 109 for testosterone propionate which was used as the internal standard. The corresponding parent ions were *m/z* 446 and *m/z* 345. A linear calibration curve was observed within the concentration range of 0.10–200 ng/ml. The lowest limit of quantitation (LLOQ) was 0.1 ng/ml. The extraction-efficiency at three concentrations was 100.7, 93.6, and 99.7%. Practical utility of this new LC–MS/MS method was confirmed in pilot pharmacokinetic studies in humans following oral administration.

Keywords: LC–MS/MS; Viaminate; Human plasma

1. Introduction

Viaminate, all-*trans*-*N*-(4-ethoxycarbophenyl)retinamide, a retinoid derivative, was synthesized from all-*trans*-retinoic acid and *p*-aminobezoic acid ethylester. The effect of viaminate on the human body is similar to that of 13-*cis*-retinoic acid which is the *cis*-stereoisomer of all-*trans*-retinoic acid. At present, viaminate (Fig. 1) is primarily used in the topical treatment of acne vulgaris which causes comedones, papules, and pustules predominate. Because the adverse effects of viaminate are less than those of other oral retinoids, viaminate has been widely used in a number of skin disorders and some forms of neoplastic disease [1–3].

Although viaminate is widely used in China, no pharmacokinetic studies of it in humans have been reported. A high-performance liquid chromatography (HPLC) method with UV detection was developed for the study of pharmacokinetics in mice [4]. However, as the concentration of viaminate is very low in plasma, it is difficult to determine it in human plasma by using UV detection. As mass spectrometry (MS) is a much more sensitive detection method than UV detection, it is more suitable for detecting the concentration of viaminate in human plasma when low doses were given orally. The purpose of this work was to develop a quantitative method for viaminate in plasma, by using a simple extraction procedure, efficient HPLC separation, and specific MS detection. To our best knowledge, this method for determination of viaminate in human plasma has not been published so far. Using the described method in this report, pilot pharmacokinetics studies of viaminate were characterized after oral administration.

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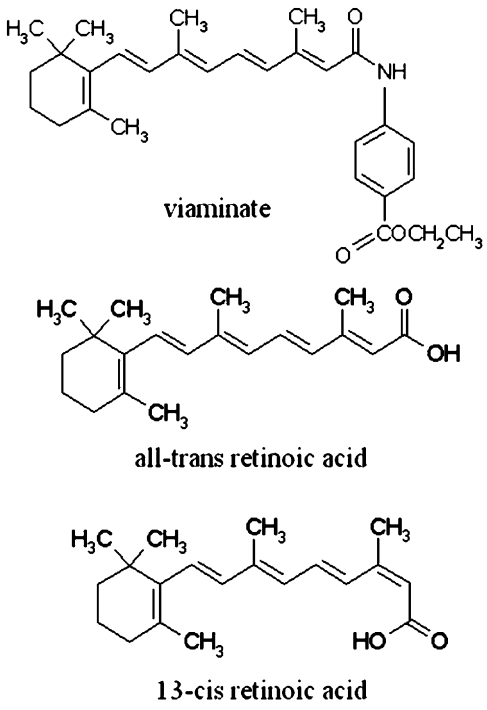

Fig. 1. Structures of viaminate, all-*trans*-retinoic acid and 13-*cis* retinoids acid.

2. Experimental

2.1. Materials and reagents

Testosterone propionate (batch No: 0008–9404), obtained from the National Institute for The Control of Pharmaceutical and Biological Products (NICPBP, Beijing, China), was used as the internal standard (I.S.) without any purification.

Viaminate standard (99.63% purity in HPLC) was supplied by Chongqing Huapont Pharmaceutical Co. Ltd. without any purification.

HPLC grade methanol was purchased from SK Chemicals (Ulsan 680-160, Korea). All other chemicals were of analytical-grade and were purchased from Nanjing Chemical Reagent Co. Ltd. (Nanjing, China). Distilled de-ionized water was produced by a Mill-Q UF Plus Water Purification Unit (Millipore, MA, USA).

2.2. Methods

2.2.1. LC-UV conditions

HPLC analysis was performed using a Shimadzu 2010 system (Shimadzu, Japan) with an SPD-10A VP UV-Vis detector (Shimadzu, Japan). Separations were achieved by using a VP-ODS column (250 mm × 4.6 mm i.d., 5 μm particle size; Shimadzu, Japan) which was protected by a GVP-ODS pre-column (10 mm × 4.6 mm i.d.; Shimadzu, Japan). The mobile phase composed of methanol–water (90:10, v/v) was used at a flow rate of 1 ml/min. The effluents were monitored at 370 nm.

2.2.2. LC–MS conditions

LC–MS analysis was performed using an Agilent 1100 Series LC–MS Trap system (Agilent, USA), and a Shimadzu 2010 LC–MS system (Shimadzu, Japan). Separations were achieved by using a VP-ODS column (250 mm × 2.0 mm i.d., 5 μm particle size; Shimadzu, Japan) which was protected by a GVP-ODS pre-column (10 mm × 2.0 mm i.d.; Shimadzu, Japan). The mobile phase composed of methanol–water (95:5, v/v) was used at a flow rate of 0.2 ml/min. The column oven was maintained at 25 °C.

The mass spectral analysis was performed in the negative-ion mode. The capillary voltage was set at 3500 V. The nebulizer gas pressure was set at 35 psi. The drying gas flow was set at 8 l/min, and the drying gas temperature was set at 350 °C. Quantification was performed by selected ion monitoring of the ion at *m/z* 446 for viaminate.

2.2.3. LC–MS/MS conditions

LC–MS/MS analysis was performed using a system consisting of a Finnigan autosampler (Thermo Electron Corperation, USA), a Finnigan LC pump, a Finnigan TSQ Quantum Ultra equipped with an electrospray ion source and operated by XCalibur software. The LC condition was the same as that in Section 2.2.2. Testosterone propionate was used as I.S. for the quantification. The total analysis time was 12 min.

The mass spectral analysis was performed in the negative-ion mode. The spray voltage was set at 5000 V. The sheath gas and AUX gas pressure were set at 35 and 5 psi, respectively. The collision gas (Ar) pressure was set at 1.5 mTorr. The capillary temperature was set at 270 °C. Collision induced dissociation (CID) studies were performed using collision energy of 25 eV. The transitions monitored were *m/z* 446 (parent ion) to *m/z* 164 (product ion) for viaminate, and *m/z* 345 (parent ion) to *m/z* 109 (product ion) for testosterone propionate. The dwell time was 100 ms for each transition.

From 0 to 4 min, the valve was switched to "waste" position, and the eluate was abandoned. Then the valve was switched to "source" position, allowing the eluate to flow to ion source for analysis. The product ions at *m/z* 109 (I.S.) and *m/z* 164 (viaminate) were monitored within the retention time windows of 4–8 and 8–12 min, respectively.

In practice, to maintain the LC–MS/MS system in excellent working condition, the pre-column filter and analytical column were cleaned once after a 24-h run, and the ESI source was disassembled and cleaned once after 1-week run.

2.3. Laboratory precautions

As viaminate is light sensitive, all experiments were done under low light level conditions with a weak yellow light source.

2.4. Preparation of stocks, calibration standards and quality control samples

The stock solutions of viaminate and I.S. were prepared in methanol at concentration of 1 mg/ml. Fresh working solutions were prepared by diluting the stock solutions in methanol to concentration of 1 μg/ml. Calibrations standards were prepared by adding different concentrations of the working solutions to drug-free plasma. The resulted final concentrations were 0.10,

0.50, 1.00, 2.00, 5.00, 10.0, 20.0, 40.0, 100, and 200 ng/ml of viaminate. Three pools of quality control (QC) samples were prepared by spiking blank plasma with the viaminate at the concentrations of 1, 10, and 100 ng/ml. All solutions were stored in brown glass volumetric flask at 4 °C before analysis.

2.5. Sample preparation

For analysis, into a 10 ml glass tube, each of 1 ml of plasma was accurately transferred, and 50 μl of I.S. working solution (1 μg/ml) and 1 ml of ethanol were added. After the glass tube was vortexed for 30 s, 4 ml cyclohexane was added. The mixture was vortexed for 2 min and then centrifuged at 1500 × *g* for 10 min. The supernatant was transferred into a second test tube and evaporated to dryness under a stream of nitrogen at 40 °C. The residual were dissolved in 200 μl of methanol, then vortexed for 2 min and centrifuged at 3000 × *g* for 10 min. Finally, 10 μl of supernatant was injected for LC–MS/MS analysis.

2.6. Method validation

Validation of this method was performed in compliance with FDA guidelines for biological method validation [5].

2.6.1. Quantification

To determine the concentration of viaminate in plasma samples, peak-area ratios of viaminate to the I.S. were calculated and related to standard curves of viaminate in drug-free plasma. The lower limits of detection (LLOD) was defined as the lowest concentration by a signal-to-noise ratio of 3; the lower limits of quantitation (LLOQ) was defined as the lowest concentration of the standard in the calibration curve by a signal-to-noise ratio of 10.

2.6.2. Extraction-efficiency

The extraction-efficiency of viaminate was determined at three QC levels by comparing the analyte peak-area ratios in spiked samples with the peak-area ratios of samples that had the analyte spiked post-extraction. The internal standards were added to both sets of samples post-extraction.

2.6.3. Linearity, precision and accuracy

The calibration standard curves were constructed using 10 non-zero standards ranging from 0.10 to 200 ng/ml. A blank sample (matrix sample processed without I.S.) was used to exclude contamination. The linearity of the relationship between peak-area ratio and concentration was demonstrated by the correlation coefficient (*R*) obtained for the linear regression. The relative standard deviation (RSD) was calculated for all slopes of calibration curves.

The intra- and inter-day assays of the method were evaluated by quintuplicate analyses of three QC samples. The calibration standards and quality controls were analyzed on 5 different days in order to determine intra- and inter-day precision and accuracy. The accepted criteria for each quality control were that the RSD should not exceed 15%.

2.6.4. Stability

The stability of viaminate and I.S. solution at room temperature were determined by leaving the plasma samples (spiked with viaminate at 100 ng/ml) at room temperature for 24 h. To evaluate long-term stability of viaminate in plasma, three plasma samples (at 100 ng/ml), were stored at −20 °C for 3 weeks. Freeze–thawed stability of viaminate in plasma was determined by subjecting plasma samples (at 100 ng/ml) to five freeze–thawed cycles (−20 °C/+25 °C) for 35 days.

2.7. Determination of viaminate in human plasma

The human experiments were approved by the Ethics Committee of the Institute of Dermatology, Peking Union Medical College & Chinese Academy of Medical Sciences according to the regulation of State Food and Drug Administration, PR China. A single oral dose of 50 mg viaminate capsule was administered to 3 healthy human volunteers. The blood samples (about 3 ml) were collected in heparinized tubes at 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15 and 24 h after oral administration. All blood samples were separated following centrifugation at 3000 × *g* for 5 min and the plasma samples were stored at −20 °C until analysis.

The concentrations of viaminate in human plasma were calculated according to the daily prepared calibration curve.

3. Results and discussions

3.1. Selection of extraction method and internal standard

In this report, the methods of liquid–liquid extraction [4,6] had been tested for extracting viaminate and I.S. from the plasma samples. It was found that the extraction efficiencies of viaminate and I.S. were rather low. Therefore, two steps of precipitating the protein and then extracting viaminate and I.S. were performed. By testing acetone, methanol, ethanol, and acetonitrile for precipitating protein, and ethyl acetate, ethyl ether, and cyclohexane for extracting viaminate and I.S., we found that the highest extraction-efficiency could be obtained by using ethanol–cyclohexane system. The extraction-efficiencies of viaminate and I.S. were 98 ± 4% and 95± 5%, respectively.

The ideal I.S. is a compound with similar structure to the analyte, but sometimes it is difficult to get such material. In this report, testosterone propionate was selected as the I.S. by virtue of similarity to viaminate with respect to extraction-efficiency, chromatographic behavior, and mass spectrometry detection.

3.2. Selection of the methodology

Because the concentration of viaminate in human plasma is low, a sensitive method for detecting viaminate is required. We used HPLC with UV detection (LC-UV) and two different LC–MS instruments for the analysis of viaminate in human plasma. The results showed that the LLOQ values of the methods were 100 ng/ml in LC-UV, 5 ng/ml in LC–MS using the Agilent apparatus, and 1 ng/ml using the Shimadzu LC–MS system.

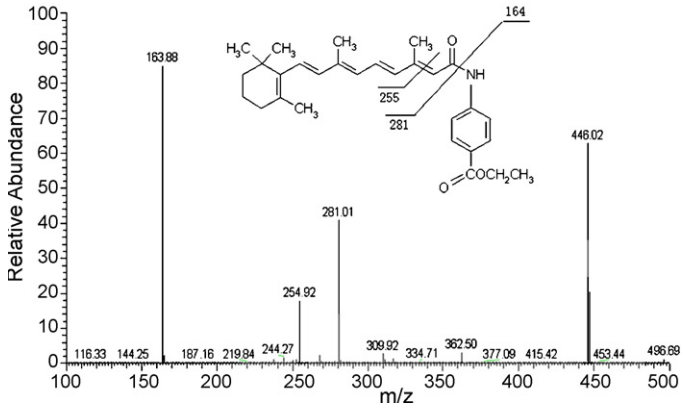

Fig. 2. LC–MS/MS spectrum for *m/z* 446 of viaminate in negative-ion mode.

Despite higher sensitivity of the LC–MS methods compared with LC-UV, the lowest LOQ value of 1 ng/ml did not fulfill the requirement for detecting viaminate in plasma samples.

As LC–MS/MS is a very sensitive detection method, we used this method for the quantitative determination of viaminate in human plasma. By using a combination of full and product ion scanning techniques, we found that $[M-H]^-$ ion (*m/z* 446) of viaminate obtained in negative-ion mode have higher abundance than $[M+H]^+$ ion (*m/z* 448) obtained in positive-ion mode. Compared with positive-ion mode, the baseline noise in negative-ion mode was lower. By monitoring the ions of *m/z* 448 (parent ion) to *m/z* 283 (product ion), the LOQ of LC–MS/MS method performed in the positive-ion mode was 2 ng/ml. On the other hand, the LOQ of LC–MS/MS method performed in a negative-ion mode was 0.1 ng/ml when ions of *m/z* 446 (parent ion) to *m/z* 164 (product ion) were monitored. Thus, the mass spectral analysis was performed in the negative-ion mode. The mass spectrum of viaminate derived from the parent ion at *m/z* 446 shows intense product ions at *m/z* 281 and *m/z* 164. Among all of these ions, the product ion at *m/z*164 had the highest abundance (Fig. 2). The LC–MS/MS spectrum and chemical structure of viaminate, and the possible fragmentation mechanism are also showed in Fig. 2.

3.3. Method validation

Under the described chromatographic conditions with a mobile phase of methanol–water (95:5, v/v), the retention time was about 5.06 min for viaminate and 10.20 min for the I.S. At the retention time, the test substance viaminate and the I.S. were eluted without any interference peaks from the blank human plasma (Fig. 3). The average extraction efficiencies of viaminate at three QC levels were from 90% to 105%

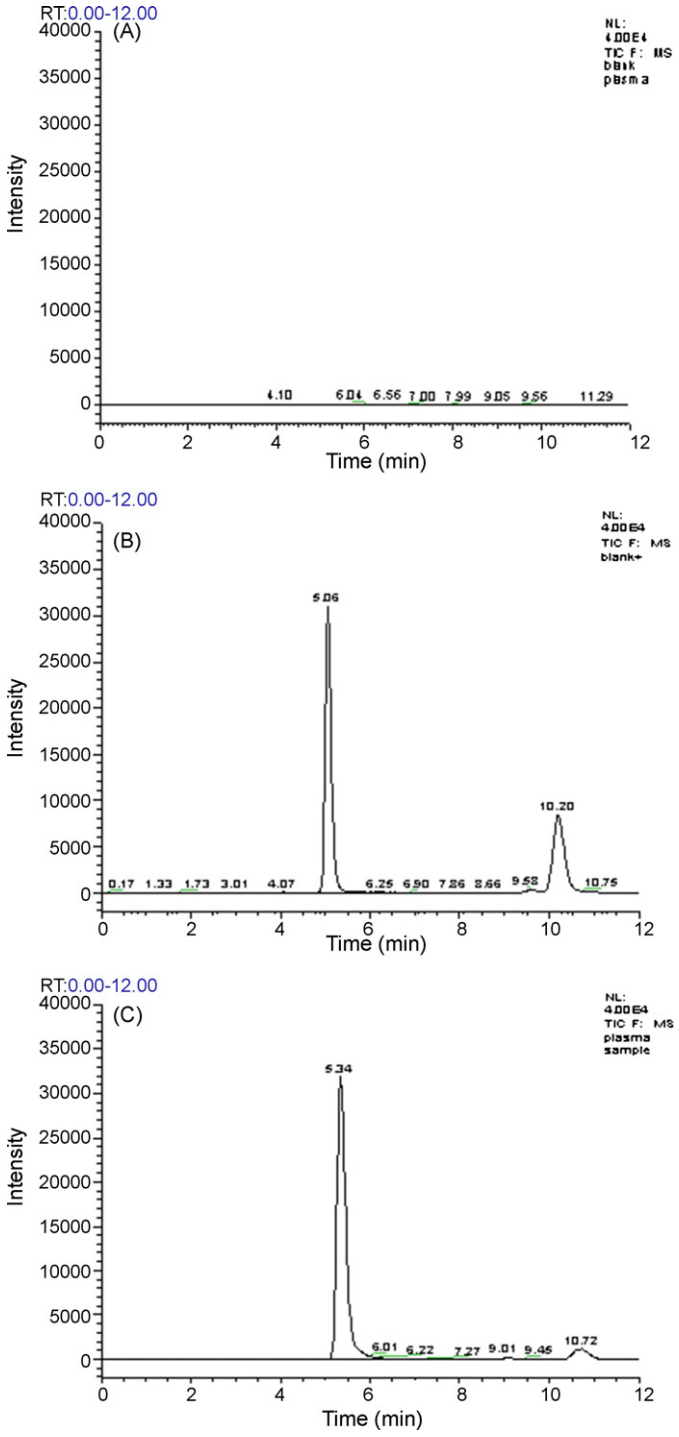

Fig. 3. LC–MS/MS chromatograms of (A) blank plasma; (B) blank plasma spiked with viaminate (10 ng/ml) and I.S. (1 μg/ml); and (C) processed plasma sample of human after administrated 50 mg viaminate capsule 3 h which contained 1.32 ng/ml viaminate.

Table 1
Extraction-efficiency of viaminate from spiked human plasma ($n=5$)

Added *C* (ng/ml)	Extraction-efficiency (%)					Found *C* (%)	RSD (%)
1	99.2	104.0	100.5	98.1	101.9	100.7	2.3
10	93.3	103.0	90.8	88.0	93.0	93.6	6.0
100	103.8	98.6	93.7	103.8	98.6	99.7	4.2

Table 2
Precision, accuracy for the determination of viaminate in human plasma (intra-day: $n = 5$; inter-day: $n = 5$ series per day, 5 days)

Added C (ng/ml)	Intra-day			Inter-day		
	Found C (ng/ml)	RSD (%)	Relative error (%)	Found C (ng/ml)	RSD (%)	Relative error (%)
1	1.07 ± 0.01	3.5	7.0	1.03 ± 0.02	5.7	−3.0
10	9.89 ± 0.17	2.9	−1.1	9.60 ± 0.33	3.5	−4.0
100	100.2 ± 4.58	4.8	0.3	96.56 ± 3.15	3.3	−3.4

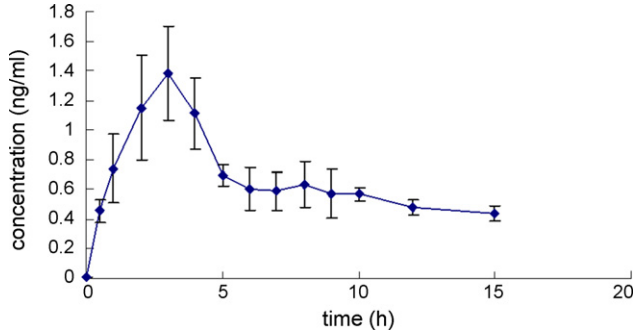

Fig. 4. Plasma concentration–time relationship of viaminate observed after administration of 50 mg dose in 3 volunteers (mean ± SD).

(Table 1). A linear relationship was found between peak-area ratio and drug concentrations within the range of 0.1–200 ng/ml for plasma samples. The calibration curves were found to be linear and could be described by the regression equations, $Y = 0.0519 \times X - 0.00453$ (5 samples of 10 different concentrations, Y: peak-area ratio, X: concentration of viaminate), with correlation coefficient of over 0.999. The RSD of slope for calibration curves was 9.3% which indicates a high precision of the assay. The LOD and LOQ of viaminate were found to be 0.04 and 0.1 ng/ml in plasma, respectively. This sensitivity has proven useful in the determination of viaminate in human plasma after oral administration.

The intra- and inter-day precision and accuracy of viaminate in human plasma is listed in Table 2. The data demonstrates that the method is reliable and reproducible since RSD was below 15% for all investigated concentrations (Table 2).

The stability of plasma samples containing viaminate and I.S. was fully evaluated by analyzing 100 ng/ml QC samples. After storage at room temperature for 24 h, viaminate concentrations in plasma deviated from the concentrations in unstored plasma by less than ±6%. When processed samples were stored at −20 °C, viaminate showed very good stability at the concentration studied; the responses varied not more than ±7% within 3 weeks of storage. The concentration variations found after five cycles of freezing at −25 °C and thawing at 25 °C were within ±5% of nominal concentrations. These results suggest that viaminate is stable in human plasma.

3.4. Determination of viaminate in human plasma

A representative chromatogram from the analysis of viaminate in a human plasma sample is shown in Fig. 2C. After single-dose oral administration (50 mg/2 caps), the plasma drug concentration–time relationship were shown in Fig. 4.

4. Conclusions

A sensitive and specific LC–MS/MS method was developed for analysis of viaminate in human plasma. The method uses a simple and high-yield liquid/liquid extraction of viaminate and the internal standard testosterone propionate from plasma, is sensitive, specific and accurate. The present LC–MS/MS method allows performance of pharmacokinetic studies of viaminate in humans.

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