Drug Consistency Evaluation of Atorvastatin Using Urine Proteomics

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Abstract

[Objective] To conduct pharmaceutical consistency evaluation of atorvastatin tablets through urine proteomics analysis.

[Methods] Rat gavage models were established for atorvastatin tablets from two different pharmaceutical manufacturers. Urine samples were collected before and after gavage, and extracted urinary proteins were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Differential proteins were further analyzed for biological pathways using the DAVID database.

[Results] Compared with pre-gavage, 116 differential proteins were identified in the drug A group gavage model, and 66 differential proteins were identified in the drug B group gavage model, with 24 proteins commonly identified between the two groups. These differential proteins tended to be involved in biological pathways such as cell adhesion, negative regulation of endopeptidase activity, and proteolysis.

[Conclusion] For atorvastatin tablets produced by different manufacturers, differences can be detected through urinary proteins, which validates the sensitivity of urinary proteins and suggests that urine proteomics has great potential in pharmaceutical consistency evaluation.

Full Text

Drug Consistency Evaluation of Atorvastatin Using Urine Proteomics

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Abstract

[**Objective**] To evaluate the drug consistency of atorvastatin tablets through urine proteomic analysis. [**Methods**] Intragastric administration rat models were established using atorvastatin tablets from two pharmaceutical manufacturers. Urine samples were collected before and after administration, and urinary proteins were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Differential proteins were subjected to biological pathway analysis using the DAVID database. [**Results**] Compared with pre-administration levels, 116 differential proteins were identified in the drug A group and 66 in the drug B group, with 24 proteins common to both groups. These differential proteins were primarily involved in biological pathways such as cell adhesion, negative regulation of endopeptidase activity, and proteolysis. [**Conclusions**] Urinary proteins can detect differences between atorvastatin tablets from different manufacturers, confirming the sensitivity of urinary protein analysis and demonstrating the substantial potential of urine proteomics for drug consistency evaluation.

Keywords: urine; proteomics; drug consistency evaluation

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Current drug consistency evaluation relies on chemical and biological methods. Chemical approaches involve measuring drug dissolution profiles, while biological methods typically involve administering drugs to healthy volunteers, collecting venous blood after fasting, and comparing relevant indicators to determine consistency. Based on these existing evaluation methods, we propose a novel approach utilizing urinary protein biomarkers for consistency assessment. The fundamental characteristic of a biomarker is its capacity for “change” —appropriate biomarkers can reflect bodily changes earlier and more sensitively. Blood, as a commonly used sample, is under strict homeostatic regulation, where most change information is eliminated by regulatory mechanisms and exists only transiently. In contrast, urine is a product of blood filtration, not strictly regulated by homeostatic mechanisms, and can accommodate and accumulate more extensive changes [**?**]. Moreover, urinary proteins remain stable for extended periods [**?**], and the relative simplicity of the urine proteome facilitates detection of lowabundance protein changes [?], making urine an excellent source for biomarker discovery. Since the urine proteome is susceptible to various factors such as diet, medication, and daily activities, employing a simple and controllable system is crucial for accurate results. Animal models are particularly suitable as their genetic and environmental factors can be controlled to minimize confounding influences. This raises the question: can we evaluate the consistency between original and generic statin drugs from a urinary perspective?

Statins are HMG-CoA reductase inhibitors widely used clinically as lipid-lowering agents [?]. Their lipid-lowering mechanism involves competitive inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the rate-limiting enzyme in cholesterol synthesis, thereby reducing intracellular cholesterol levels. This mechanism also upregulates low-density lipoprotein receptor expression on hepatocytes, regulating blood lipids [?]. Additionally, statins exhibit pleiotropic effects beyond lipid modulation, playing important roles in treating atherosclerosis, coronary heart disease, atrial fibrillation, ventricular arrhythmias, and cardiac hypertrophy [?]. In this study, we administered appropriate doses of two atorvastatin tablets to rats via intragastric gavage, collected urine before and after administration, and performed label-free quantitative proteomic analysis to investigate and compare proteomic changes induced by the two drugs, thereby providing an innovative approach for drug consistency evaluation.

2.1 Establishment of Intragastric Administration Animal Models

Ten healthy male Sprague-Dawley rats (160$±20$g)werepurchasedfromBeijingVitalRiverLaboratoryAnimalT
humidity 65%-70%) for three days before experimentation. All procedures were reviewed and approved by the Ethics Committee of the College of Life Sciences, Beijing Normal University.

The intragastric administration model was established as follows: using a self-controlled before-and-after design, ten rats were randomly divided into groups A and B (n=5 each). Group A received drug from manufacturer A dissolved in saline, while group B received drug from manufacturer B dissolved in saline. Both drug formulations were identical, administered at 10 mg/kg once daily for five consecutive days. Rat body weight was measured daily to adjust drug dosage accordingly.

2.2 Urine Sample Collection

Prior to drug administration, rats were placed in metabolic cages for 12-hour urine collection. After three days of standard housing, intragastric administration was performed once daily for five consecutive days. On day 5, following the final administration, rats were again placed in metabolic cages for 12-hour urine collection. During collection periods, rats were deprived of food and water. All urine samples were stored at -80°C.

2.3 Urine Sample Processing

Urinary Protein Extraction and Quantification: Urine samples collected at two time points were centrifuged at 12,000×g for 40 minutes at 4°C. Supernatants were transferred to new EP tubes, and three volumes of pre-cooled ethanol were added. After thorough mixing, proteins were precipitated overnight at -20°C. The following day, ethanol supernatants were centrifuged at 12,000×g for 30 minutes at 4°C. Supernatants were discarded, protein pellets were retained, inverted onto filter paper, and dried with cold air from a hair dryer. Protein pellets were resuspended in 120 L lysis buffer (8 mol/L urea, 2 mol/L thiourea, 25 mmol/L dithiothreitol, 50 mmol/L Tris) by repeated pipetting until completely dissolved, followed by vortex mixing for two hours. After complete dissolution, samples were centrifuged at 12,000×g for 30 minutes at 4°C. Supernatant proteins were transferred to new EP tubes, and protein concentration was measured using the Bradford method.

Urinary Protein Digestion: 100 g of urinary protein was loaded onto a 10 kDa ultrafiltration tube filter membrane (Pall, Port Washington, NY, USA) placed in a 1.5 mL centrifuge tube. 25 mmol/L NH_4HCO_3 solution was added to a total volume of 200 L, followed by 20 mM dithiothreitol (DTT, Sigma). After vortex mixing, samples were heated at 97°C for 5 minutes in a metal bath and cooled to room temperature. 50 mM iodoacetamide (IAA, Sigma) was added, mixed, briefly centrifuged, and incubated at room temperature in darkness for 40 minutes. Membrane washing was then performed: (1) 200 L UA solution (8 mol/L urea, 0.1 mol/L Tris-HCl, pH 8.5) was added and centrifuged at 14,000×g for 5 minutes at 18°C, repeated twice; (2) samples were loaded and centrifuged at 14,000×g for 40 minutes at 18°C; (3) 200 L UA solution was added and centrifuged at 14,000×g for 40 minutes at 18°C, repeated twice; (4) 25 mmol/L NH_4HCO_3 solution was added and centrifuged at 14,000×g for 40 minutes at 18°C, repeated twice; (5) trypsin (Trypsin Gold, Promega, Fitchburg, WI, USA) was added at a 1:50 enzyme-to-protein ratio for overnight digestion at 37°C. Finally, peptides were desalted using HLB columns (Waters, Milford, MA), vacuum-dried, and stored at -80°C.

2.4 LC-MS/MS Tandem Mass Spectrometry Analysis

Digested samples were resuspended in 0.1% formic acid. Peptide concentration was quantified using a BCA kit and diluted to 0.5 g/ L. For each sample, 9 L was pooled to create a mixed peptide sample. High pH reverse-phase peptide fractionation was performed using a kit (Thermo Fisher Scientific) according to the manufacturer' s instructions. Ten fractions were collected by centrifugation, vacuum-dried, and resuspended in 0.1% formic acid. iRT standard (Biognosis) was added at a 10:1 sample-to-iRT volume ratio. Each sample (individual experimental samples and ten fractions) was analyzed by mass spectrometry using an EASY-nLC1200 chromatography system (Thermo Fisher Scientific, USA) and Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, USA).

2.5 Data Processing

Raw files from the ten fractions were analyzed using Proteome Discoverer 2.1 (PD) software to establish a DIA acquisition method. The newly established DIA method was used for single-sample DIA mode acquisition. Spectronaut X software was employed for processing and analyzing mass spectrometry data. Raw files from each DIA acquisition were imported for database searching. Protein quantification was performed using the peak area of all fragment ions from secondary peptides.

2.6 Data Analysis

Using a self-controlled design, proteins identified before and after intragastric administration were compared to screen for differential proteins. Screening criteria were: fold change \$ \$1.5 or \$ \$0.67, and adjusted P-value <0.05 from paired t-test analysis. Differential proteins were subjected to biological analysis using the UniProt website (https://www.uniprot.org/) and DAVID database (https://david.ncifcrf.gov/). Published literature was searched in PubMed for functional analysis of differential proteins.

3.1 Behavioral Analysis of Experimental Animals

In this study, behavioral observations were conducted on rats in groups A and B before and after model establishment. Both groups exhibited normal activity, feeding, and drinking behaviors with no significant behavioral differences between groups. Body weight increased continuously in both groups during the five-day administration period, indicating normal growth. Detailed weight changes are shown in Figure 1 [Figure 1: see original paper].

3.2 Analysis of Urinary Proteome Changes

(1) Urinary Protein Identification: Following intragastric administration, 20 urinary protein samples (before and after administration) from both groups were analyzed by LC-MS/MS. A total of 1,261 proteins were identified (\$ \$2 unique peptides, protein-level FDR<1%). Comparing post-administration urine with pre-administration urine in groups A and B, differential proteins were screened using criteria of fold change \$ \$1.5 or \$ \$0.67 and two-tailed unpaired t-test P<0.05. Results showed 116 differential proteins in group A and 66 in group B. Detailed information on differential proteins is listed in Table 1 (group A) and Table 2 (group B). A Venn diagram illustrates the overlap of differential proteins identified after administration of the two drugs (Figure 2 [Figure 2: see original paper]), showing 24 proteins common to both groups. Partial least squares-discriminant analysis (PLS-DA) of total proteins is shown in Figure 3 [Figure 3: see original paper].

(2) Functional Analysis of Differential Proteins in Group A: DAVID database analysis of differential proteins in group A revealed enrichment in biological processes, cellular components, and molecular functions (Figure 4 [Figure

4: see original paper]). These differential proteins were primarily involved in cell adhesion, substrate adhesion-dependent cell spreading, negative regulation of endopeptidase activity, positive regulation of peptidyl-tyrosine phosphorylation, cell migration, and proteolysis. In terms of cellular components, most differential proteins originated from extracellular regions and the outer plasma membrane. Molecular function analysis revealed enrichment in integrin binding, hydrolase activity, serine-type endopeptidase inhibitor activity, and insulin-like growth factor binding.

(3) Functional Analysis of Differential Proteins in Group B: Similarly, functional analysis (Figure 5 [Figure 5: see original paper]) showed that differential proteins in group B were primarily involved in proteolysis, hepatocyte growth factor-stimulated cell response, immune response, cysteine metabolism, thyroid hormone-stimulated cell response, and glutathione metabolism. Cellular component analysis indicated these proteins originated from extracellular regions and secretory vesicles. Molecular functions included peptidase activity, carboxypeptidase activity, hydrolase activity, endopeptidase inhibitor activity, and glutamate-cysteine ligase activity.

4 Discussion

This study established rat intragastric administration models using atorvastatin tablets from two manufacturers and investigated their effects on urinary proteins through label-free LC-MS/MS analysis of pre- and post-administration urine samples. Statistical analysis revealed 116 differential proteins in group A and 66 in group B, with 24 proteins common to both groups.

Partial least squares-discriminant analysis demonstrated that urinary proteins can reveal significant effects of atorvastatin on the organism, and differences between the two drugs from different manufacturers with identical composition, formulation, and dosage could be observed, indicating that urinary proteins can capture subtle changes. However, functional analysis of differential proteins did not clearly distinguish whether these effects originated from pharmacological or toxicological actions. Notably, we did not observe significant lipid-lowering-related biological pathways. Furthermore, statin side effects might be expected to manifest as pathways related to smooth muscle cell migration and platelet adhesion downregulation [**?**], which were not observed in this study. At the current research stage, the absence of significant pharmacological or toxicological effects may be attributed to dosage issues. Nevertheless, even at low doses, we could still observe organismal effects through urine analysis, demonstrating the remarkable sensitivity of urine and its substantial potential as a method for drug consistency evaluation.

5 Conclusion

Through establishment of an atorvastatin rat intragastric administration model, we could significantly distinguish drug effects via urinary proteins. Urinary proteins could even detect subtle differences between atorvastatin tablets from different manufacturers, fully validating the sensitivity of urine and suggesting great potential for urine proteomics in drug consistency evaluation.

Table 1 Differential proteins identified in group A

Table 2 Differential proteins identified in group B

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