

Chapter 2

Urine Reflection of Changes in Blood

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Abstract The most important of nature of biomarker is changes. Blood is under strict homeostatic control which means changes tend to be removed from blood. Urine is a partial filtrate of blood, reflects systemic physiology but with no homeostatic mechanism. However, changes induced directly into the blood can be more sensitively detected in urine than in blood itself. This indicates that urine may serve as a source for more sensitive detection of protein biomarkers than blood.

Keywords Change · Biomarker · Urine · Blood

Theoretically, urine may be a better source for biomarker discovery than plasma. Given that change is an essential property of a biomarker, the strict homeostatic regulation of plasma composition would in theory act to remove any changes in blood. While urine collects all wastes from the body, it can accumulate more and bigger changes [1]. Therefore, urine serves not only as an ideal source of biomarker discovery for diseases of the kidney and other tissues of the urogenital system but also as a potential source of information on diseases in other physiological systems.

In comparison to plasma, urine has many other advantages as a source for biomarker discovery. First, urine can be collected noninvasively, frequently, and in large quantities. Second, while plasma is generally obtained at a single time point, multiple urine samples can be collected over a period of time, allowing for an easier monitoring of time-dependent changes in biomarker levels. Third, proteolytic degradation may be complete prior to collection of urine [2] and because proteases are activated during blood collection [3], the urinary proteome shows much greater stability compared with that of plasma. Urinary proteins, for example, show no significant changes when urine is stored for three days at 4 °C or for 6 h at room

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temperature [4]. The use of membranes to store urinary proteins is simple and economical and facilitates biomarker research by making large-scale storage of clinical urinary samples possible [4].

In paper published on Science China Life Science [5], two different anticoagulants were used (unfractionated heparin and argatroban) to disrupt the blood coagulation status of adult female SD rats. Changes in urine and plasma proteomic profiles in response to treatment with the drugs were compared with exactly same standard to observe whether there are more changes in urine.

Six adult female Sprague-Dawley rats were initially anesthetized by an intravenous injection of 20 mg kg⁻¹ pentobarbital sodium, after which a catheter was inserted for urine sampling.

Saline (0.9 % NaCl in sterile water) was first administered via the great saphenous vein as a single bolus with a volume equivalent to that of the anticoagulants. Urine was collected over the following 30 min as a control specimen. Rats then received a bolus of argatroban (2 mg kg⁻¹) the same vein, after which urine was collected over the following 30 min. Only rats that showed no gross hematuria were considered to be successful preparations and proceeded to the next stage. Urine was centrifuged at 3,000 g for 10 min at 4 °C. After removal of precipitates, urinary proteins were extracted by acetone precipitation [6] and subjected to quantitation by the Bradford method.

Venous blood was collected by aspiration from the femoral vein at three time points: 5 min after saline injection and 5 min and 30 min after administration of anticoagulants. For the assessment of clotting time (CT), 25 µL of the blood sample was used. The remaining blood sample was immediately transferred to a plastic tube containing 0.109 M sodium citrate at a ratio of 1:9, gently mixed and centrifuged at 2,000 g at 4 °C for 10 min to obtain citrated plasma. Plasma was stored at -80 °C until further analysis.

Heparin (5 mg/kg) was given intravenously to another group of rats by the same procedure as argatroban experiment described above.

The identical proteomic analysis approach was used to analyze the protein composition of rat urine and plasma samples before and after treatment with anticoagulants. Software Progenesis were used to perform label-free quantification. Briefly, for protein identification, the false positive rate was 1 %. For quantification, only unique peptides and proteins with at least two peptides were included. Besides, the direction of change in each animal should be consistent and the fold change should be ≥1.5 in at least one animal.

LC-MS/MS analysis detected consistent heparin-induced changes in the levels of 27 proteins in urine but only three proteins in plasma (Table 2.1) in three drug-treated rats and three control rats. There was no overlap between these groups of proteins. Consistent changes in the levels of 61 proteins were detected in urine in response to treatment with argatroban, only one of which changed in plasma. The number of proteins identified in plasma was much smaller than the number detected in urine. These results indicate that changes in protein levels may be more sensitively detected using LC-MS/MS in urine than in plasma.

Table 2.1 Changed proteins in heparin-treated group [5]

Accession	Max fold change in urine	Change tendency in urine	Max fold change in plasma	Change tendency in plasma	Description
ITIH3_RAT	2.5	up	N		Inter-alpha-trypsin inhibitor heavy chain H3
CO4_RAT	2.8	up	N		Complement C4
IGG2C_RAT	3.1	up	N		Ig gamma-2C chain C region
FIBB_RAT	2.1	up	N		Fibrinogen beta chain
HEMO_RAT	2.6	up	N		Hemopexin
FIBG_RAT	3.4	up	N		Fibrinogen gamma chain
AFAM_RAT	3.7	up	N		Afamin
APOA1_RAT	3.4	up	N		Apolipoprotein A-I
FETUB_RAT	2.9	up	N		Fetuin-B
CADH1_RAT	1.5	down	N		Cadherin-1
ALBU_RAT	3.1	up	N		Serum albumin
FETUA_RAT	2.9	up	N		Alpha-2-HS-glycoprotein
APOH_RAT	2.4	up	N		Beta-2-glycoprotein 1
EGF_RAT	2.3	down	N		Pro-epidermal growth factor
FIBA_RAT	2.4	up	N		Fibrinogen alpha chain
TRFE_RAT	2.5	up	N		Serotransferrin
CO3_RAT	2.7	up	N		Complement C3
UROK_RAT	3.5	down	N		Urokinase-type plasminogen activator
DNAS1_RAT	2.3	down	N		Deoxyribonuclease-1
IL4RA_RAT	1.8	down	N		Interleukin-4 receptor subunit alpha
KLK7_RAT	2.0	down	N		Glandular kallikrein-7
SPA3 N_RAT	2.3	up	N		Serine protease inhibitor A3N
KLK1_RAT	2.1	down	N		Kallikrein-1
APOA4_RAT	3.3	up	N		Apolipoprotein A-IV
MEP1A_RAT	5.1	down	N		Meprin A subunit alpha
PLMN_RAT	3.7	up	N		Plasminogen
6PGL_RAT	2.5	down	N		6-phosphogluconolactonase
IC1_RAT	N		1.6	down	Plasma protease C1 inhibitor
HBA_RAT	N		4.1	up	Hemoglobin subunit alpha-1/2
HBB1_RAT	N		3.9	up	Hemoglobin subunit beta-1

N indicates that the protein was not detected in the sample or failed to fulfill the quantitation criteria

Functional analysis of these differential proteins was performed by the Ingenuity Pathway Analysis (IPA) tool (<http://www.ingenuity.com/>, Ingenuity Systems, Redwood City, CA, USA). This analysis was focused on the pathways and disease mechanisms in which the proteins are expected to be involved.

The differential proteins of anticoagulants-treated rats take part in acute phase response signaling, LXR/RXR activation, coagulation system intrinsic prothrombin activation pathway and extrinsic prothrombin activation pathway. The proteins involved in coagulation pathway included F2, FGA, FGG, FGB, KLKB1, PLAUI, PLG, and SERPINA 1.

Additional six animals were treated with anticoagulants, three for each, to validate LC-MS/MS analysis of changes in the plasma and urine by Western blot. Transferrin and hemopexin were selected for validation because of the abundances and availability of antibodies. In accordance with changes in the levels of these proteins observed via the LC-MS/MS analysis, changes in the levels of both proteins in response to anticoagulant treatment were observed in urine but not in plasma (Fig. 2.1).

Biological fluids such as blood, urine, and CSF serve as common sources of biomarkers, of which plasma is most routinely used [7]. Although plasma perfuses all tissues of the body and theoretically it can collect all the information of disease biomarkers, the role of homeostatic mechanisms in eliminating changes in these biomarkers should not be ignored. Changes in blood biomarker levels are unlikely to persist long enough for detection because they are disturbances of homeostasis.

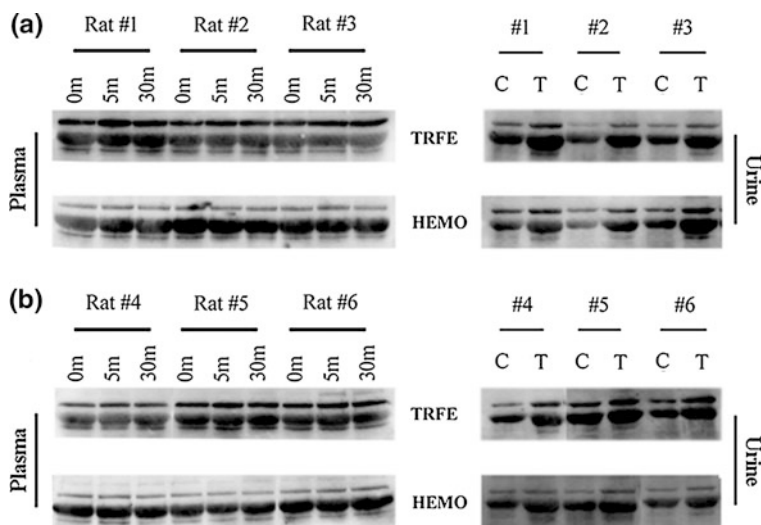


Fig. 2.1 Western blot analysis of transferrin and hemopexin in urine and plasma. 20 μ g/lane of urine or plasma proteins was loaded into gels and analyzed using Western blot. Changes in the levels of transferrin were comparable with those detected by LC-MS/MS analysis. **a** heparin-treated group. **b** argatroban-treated group. C, before anticoagulants treated; T, after anticoagulants treated [5]

Because homeostasis helps to eliminate or excrete changes in biomarker levels into urine, exhaled air, bile, and perspiration, these places may serve potential sources for biomarker discovery.

But there are exceptions. For instance, it is better to detect biomarkers in plasma when they are continuously secreted into blood or have a long half-life. Moreover, biomarkers for acute illnesses such as acute necrotizing pancreatitis (ANP) and acute myocardial infarction (AMI) are excreted into blood in large amounts in a short period of time. Plasma analysis may be more helpful to monitor progression of diseases.

There are several possible reasons that more proteins could be identified in urine than in plasma.

Firstly, any change that is introduced into the blood either internally or externally tends to be cleared by the liver, kidney, and/or other organs via a variety of mechanisms in order to maintain the homeostasis of the blood. In contrast, urine is the place that most of the wastes in blood are dumped into, and thus tolerates changes to a much higher degree. Biomarkers are the measurable changes associated with a physiological or pathophysiological process. Therefore, they are more likely to be magnified and detectable in urine than their counterparts in blood.

Secondly, for proteomics and protein biomarker experiments, high dynamic range of protein concentrations in human plasma leads to a tremendous analytical challenge. Plasma proteins have a high dynamic range, spanning at least 10^9 [8]. Albumin constitutes about half of the plasma proteins. In contrast, many potential biomarkers in plasma are at very low abundance. High-abundance proteins limit the identification of low-abundance proteins by LC-MS/MS.

Thirdly, urine as a result of being a filtrate of plasma is relatively simple in composition, the dynamic range of urinary proteins is about 10^6 [9]. Accumulated changes in urine composition are not likely to be masked, and some molecular species which are difficult to detect in blood may be detected in urine. So, more proteins would be identified in urine with the same experimental sensitivity.

Although methods such as multidimensional separation [10] can detect minor proteins or depletion of predominant proteins in plasma, they have several drawbacks. For example, these approaches can introduce additional variability; moreover, because some peptides and proteins bind to certain high-abundance proteins [11], the depletion strategy may lead to a significant loss of information.

While technological limitations prevented the detection of many components of urine in the past, more than 2,300 urinary proteins have been recently identified using high accuracy mass spectrometry [12], and technologies such as CE and SELDI-TOF MS have stimulated research into urinary biomarkers. In addition, urinary proteins containing posttranslation modifications (PTMs) such as glycosylation [13] and phosphorylation [14] are potentially rich sources of disease biomarkers [15]. Moreover, profiling of urine peptides and metabolites also reveals potential for the identification of biomarkers for systemic diseases [16]. Despite this evidence, the importance of urine serving as a biomarker source remains underestimated. For example, we retrieved 316,849 articles in a July 2013 search of PubMed using the keywords [(‘blood’ OR ‘serum’ OR ‘plasma’) and ‘biomarker’],

compared with 33,930 articles found using [(‘urine’ OR ‘urinary’) and ‘biomarker’]. In reality, the number of articles related to protein biomarkers in urine is no more than 450 in a manually organized database (until June 2013).

There have already been some data, which indicates that certain urine biomarkers are of higher quality than those from plasma. For example, urine angiotensin levels have been shown to strongly correlate with the renal pathology chronicity index [17], and urinary levels of ADAM 12 and MMP-9 have been used as noninvasive biomarkers in identifying women at increased risk of developing breast cancer [18]. Moreover, elevation in urinary desmosine levels has been shown to be associated with COPD [19].

Because urine accumulates a significant number of changes, urine samples show a greater degree of variability, particularly in protein concentration and volume [20], even though levels of constitutive proteins remain stable [21]. Such variability can be readily normalized using levels of creatinine [22], cystatin C [23], and N-acetyl- β -D-glucosaminidase (NAG) [24]. A significant challenge for biomarker discovery in urine remains discerning which changes in urine composition were caused by which systematic factor. Questions such as this can be addressed by large-scale population studies to survey normal variations in urine composition, which will lay the foundation for biomarker discovery in urine.

Blood coagulation status is a critical physiological parameter, alterations of which can lead to embolism or hemorrhage. Because the use of blood samples to monitor coagulation status is harmful and invasive, particularly in patients with high risk of spontaneous bleeding, urine samples offer an alternative approach for monitoring the coagulation state that can greatly benefit these patients.

In conclusion, using an identical proteomic analysis approach to profile the protein composition of rat urine and plasma samples before and after treatment with anticoagulants, we found that changes in the abundance of many proteins was consistently detected in urine samples but not in plasma. Urine may be the better source for biomarker discovery, because it accumulates changes, and can be collected noninvasively in large volumes. Faster and better biomarker discovery may lead to more accurate diagnosis and better health care.

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