Regular Article

Interaction of Endogenous Compounds in Human and Rat Urine with P-glycoprotein

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Summary: The effect of hydrophobic extracts of human and rat urine on in-vitro P-glycoprotein (P-gp) function was examined, in terms of intra-, inter-individual variations, and physiological states. Six urine samples out of 7, obtained from one male subject on different days, suppressed P-gp function with different potencies. Similarly, 11 samples out of 15 different individuals (8 males and 7 females) inhibited P-gp function. Among them, urine from one female, obtained 1 month after delivery, showed a potent inhibitory effect. Another urine from a pregnant female, obtained 1 week before delivery, showed further potent inhibition on P-gp function. In addition, urine from normal rats strongly inhibited P-gp function at much lower concentrations than human urine, and the inhibitory potencies varied in diseased states; control (without urine extract) = experimental acute renal failure < experimental acute hepatic failure < normal rat urine. When human urine extract was separated by a two-dimensional thin-layer chromatography, several spot fractions inhibited P-gp function, and equilin was identified in one fraction as an endogenous P-gp inhibitor. In conclusion, it was demonstrated that urine contains many endogenous P-gp inhibitors, and their inhibitory potencies on P-gp function vary with intra- and inter-individual variations, and under different physiological states.

Key words: P-glycoprotein; endogenous P-glycoprotein inhibitor; hydrophobic urine extract; human urine; rat urine; diseased states

Introduction

P-Glycoprotein (P-gp), an ATP-dependent efflux pump, is expressed in various normal tissues, including the intestine, liver, kidney, eye, brain, and so on. P-gp transports a variety of structurally and pharmacologically unrelated hydrophobic endogenous and exogenous compounds to prevent the entry and/or accumulation of P-gp substrates in tissues. Many clinically important drugs such as immunosuppressive agents, calcium channel blockers, anticancer agents and β-blockers are P-gp substrates, and expression level and/or function of P-gp affect their pharmacokinetics and pharmacodynamics. P-gp is now recognized as an important host defense mechanism for a living body, along with a variety of metabolic enzymes.

Recently, we investigated the expression and function of P-gp in rats with glycerol-induced acute renal failure (ARF) and with carbon tetrachloride (CCl₄)-induced acute hepatic failure (AHF). In these diseased rats, the in-vivo P-gp function in the liver, kidney and brain was all suppressed, even though the expression level of P-gp remained unchanged, or rather increased in the target intoxicated organs (the kidney of ARF rats and the liver of AHF rats). In Caco-2 cells, the plasma obtained from diseased rats exhibited a greater inhibitory effect on P-gp function than did plasma from normal rats. A higher plasma level of corticosterone, an endogenous P-gp substrate/inhibitor, was also observed in these diseased rats. These results suggested that concentrations and/or compositions of endogenous P-gp-related substances in plasma vary under diseased...
states and such alteration affects P-gp function systemically.

In the present study, we evaluated whether human and rat urine, as well as rat plasma, exhibit inhibitory effects on P-gp function, based on the assumption that endogenous P-gp substrates/inhibitors are excreted into the urine under normal conditions, and their excretion would be altered under different physiological states, including intra- and inter-individual variations. Evaluation was made by measuring the inhibitory effect of hydrophobic extracts of these biological fluids on transepithelial transport or accumulation of a P-gp substrate (rhodamine 123 (Rho123) or [3H] daunorubicin (DNR)) in Caco-2 or LLC-GA5-COL150 cells. Caco-2, a human colonic adenocarcinoma cell line expressing various functions of differentiated intestinal epithelial cells, is a useful in-vitro system for studying the function of P-gp in the intestine.6–9) LLC-GA5-COL150 is a transfectant cell line of a porcine kidney epithelial cell line LLC-PK1 with human MDR1 cDNA, which overexpresses human P-gp on the apical membrane.10,11) We also attempted to isolate and identify new P-gp inhibitor(s) in human urine extract.

Materials and Methods

Materials: Rhod23 was obtained from Kanto Chemicals Co. (Tokyo, Japan). Verapamil hydrochloride and 17β-estradiol were from Wako Pure Chemicals (Osaka, Japan). Equin, estriol and estron were from Sigma Chemicals Co. (MO, USA). Tritium labeled daunorubicin ([3H]daunorubicin, 185 GBq/mmol, [3H]DNR) was from Du Pont-New England Nuclear-Research Products (MA, USA). Cell culture medium and reagents including Dulbecco's modified Eagle's medium (DMEM) and Medium 199 were from Gibco Laboratories (Life Technologies Inc., NY, USA), and fetal bovine serum (FBS) from Daiichi Kagaku (Tokyo, Japan). Silica gel (Kieselgel 60 GF254) and C18 solid-phase extraction cartridges Bond ElutTM were from Merck (Darmstadt, Germany) and Varian (CA, USA), respectively. All other chemicals used were of the highest purity available.

Preparation of hydrophobic extracts of biological fluids: Bond ElutTM (Varian) was used to prepare hydrophobic extract from human urine. Permission for using urine was obtained from each healthy subject (total 16 subjects, 8 male and 8 female) with written informed consent (except for 2 little girls whose permission was obtained from their mothers). Freshly collected 100-mL human urine was applied on a cartridge of Bond ElutTM. After passing urine, the cartridge was washed out with 10-mL distilled water to remove hydrophilic substances. Substances retained in the cartridge were eluted with 1-mL methanol to obtain hydrophobic extract. The solvent was evaporated to dryness under reduced pressure, and the residue was stored at −30°C until use. Prior to experiments, the residue was dissolved with an appropriate amount of dimethylsulfoxide (DMSO). For thin-layer chromatography (TLC) study, human urine was extracted with ethylacetate to obtain a large amount of urine extract, after confirming that components of ethylacetate extract, but not of hexane or heptane extract, are close to those extracted with Bond ElutTM in a two-dimensional TLC (described below).

For preparation of rat urine and plasma extracts, male Wistar rats (260–320 g) were used. Experimental acute renal failure (ARF) and acute hepatic failure (AHF) were induced in the same manner as described previously.3,4,12) Briefly, ARF was induced by an injection of glycerol dissolved in saline (50% v/v, 10 mL/kg) into the leg muscle after a 24-h period of water deprivation.3,12) AHF was induced by an intraperitoneal injection of a mixture of carbon tetrachloride (CCl4) and olive oil (50% v/v) at a dose of 5 mL/kg.4) These rats were used for experiments 24 h after injection, and freshly collected rat urine and plasma were extracted with ethylacetate. Animal experiments were performed in accordance with the Guide for Animal Experimentation from the Committee of Research Facilities for Laboratory Animal Sciences, Graduate School of Biomedical Sciences, Hiroshima University.

Separation of human urine extract by two-dimensional TLC: Slurry of silica gel (Kieselgel 60 GF254 suspended in distilled water) was spread at a 0.5-mm layer on 20 × 20-cm plates and allowed to air dry. The plates were activated for 1 h at 120°C, and DMSO solution of human urine extract prepared from 100-mL urine was applied to the origin of a TLC plate immediately after the plate had been cooled to room temperature. The developing solvents used were a mixture of chloroform and acetone (2:1, v/v) for the first dimension and a mixture of chloroform and methanol (97:3, v/v) for the second dimension. Urine components separated on the plate were detected by UV lamp, and each spot was carefully scraped off into a tube. The silica gel containing urine component(s) was re-extracted with a sufficient amount of ethylacetate, and the solvent was evaporated, then the residue was dissolved in DMSO. These separated components of human urine extract by TLC were subjected to the study of the inhibitory effect on P-gp function or isolation/identification of new endogenous P-gp inhibitor(s).

Isolation and identification of endogenous P-gp inhibitor(s): Several spots observed in a two-dimensional TLC for human urine extract were further separated and identified by gas chromatography-mass spectrometry (GC-MS). GC-MS used was Hewlett Packard Model 5890 Series II gas chromatograph interfaced to a Hewlett Packard Model 5971 mass selective detector.
(Palo Alto, CA, USA). The capillary column used was HP-1 (0.25 mm X 30 m, Hewlett Packard), and column temperature was adjusted at 70°C (2-min hold) and increased by 15°C/min up to 270°C. Injection and detection temperatures were 250°C and 280°C, respectively.

**Cell culture:** Cells were cultured on Transwell™ cell culture chambers (Costar, Cambridge, MA). Caco-2 cells were cultured with DMEM containing 10% FBS, 1% nonessential amino acids, 2 mM L-glutamine, 100 units/mL penicillin G and 100 μg/mL streptomycin in an atmosphere of 5% CO₂-95% air at 37°C. Fresh medium was replaced every 2 days. The passages of cells used were between 67 and 86. LLC-GA5-COL150 cells were cultured with Medium 199 containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 150 ng/mL colchicine in an atmosphere of 5% CO₂-95% air at 37°C. Fresh medium was replaced every 2 days. Prior to the transport and/or accumulation studies, cells were incubated for 6 hrs with fresh Medium 199 to remove colchicine. The passages of cells used were between 7 to 24.

**Transport and accumulation studies in vitro:** Transepithelial transport and accumulation of Rho123 or [³H]DNR in Caco-2 or LLC-GA5-COL150 cells were measured in Transwell™ chamber at 37°C in the same manner as reported previously. Caco-2 cells were used at 16–21 days and LLC-GA5-COL150 cells at 5–7 days after seeding. Rho123 or [³H]DNR was dissolved in Hank’s balanced salt solution containing 25 mM HEPES, 25 mM glucose (HBSS, pH 7.4) and 0.5% DMSO, and was placed either on the apical (1.5 mL) or basolateral (2.6 mL) side of cells. The other side was filled with HBSS containing 0.5% DMSO in control study. In inhibition study, urine extract, plasma extract, or verapamil (a P-gp inhibitor) was added to both sides to give a designated final concentration with 0.5% DMSO. In studies with 17β-estradiol, equilllin, estriol and estrone, the concentration of DMSO in the medium was adjusted at 2%. After incubation for a designated period of time, the medium on the other side was periodically collected, and the concentration of a P-gp substrate was determined as described below. After transport study, the chamber was washed with a sufficient amount of ice-cold HBSS, and the cell monolayers were separated together with its support (polycarbonate membrane filter) to determine the accumulation of the substrate in cells. Cell monolayers were incubated in 0.5% trichloroacetic acid solution to release Rho123 or in 0.1 M NaOH to release [³H]DNR.

**Analyses of Rho123 and [³H]DNR:** The concentration of Rho123 in aqueous samples was determined by fluorescence spectrophotometer (Hitachi F-3000, Tokyo, Japan) at wavelengths of 485 nm for excitation and 546 nm for emission. The radioactivity of the collected medium and solubilized cell monolayers containing [³H]DNR was determined in 3 mL of a scintillation cocktail (NCSII, Amersham International, Buckingham-shire, UK) by a liquid scintillation counter (LCS-5100, Aloka, Tokyo). Differences among group mean values were assessed by Student’s t test. A difference of less than p < 0.05 was considered statistically significant.

**Results**

**Evaluation system for P-gp inhibitors:** First, several evaluation systems for P-gp inhibitors were examined by combining Caco-2 or LLC-GA5-COL150 cells as P-gp expressing cells, Rho123 or [³H]DNR as a P-gp substrate, transport or accumulation study as an experimental design, and verapamil as a P-gp inhibitor. In LLC-GA5-COL150 cells, the basal (b)-to-the apical (a) transports of Rho123 and [³H]DNR were higher than those in opposite directions, and verapamil inhibited their efflux transports (data not shown). However, the contribution of P-gp-mediated transport was much greater for [³H]DNR than for Rho123, as evaluated by the difference between b-to-a and a-to-b transports and the inhibitory effect of verapamil on the efflux transport. Similarly, verapamil increased the accumulation of [³H]DNR in LLC-GA5-COL150 cells in a greater magnitude than that of Rho123 (6.5-fold of control in [³H]DNR and 2.7-fold in Rho123). These results indicate that [³H]DNR is more sensitive than Rho123 to verapamil in LLC-GA5-COL150 cells in both transepithelial transport and accumulation studies. Based on our previous and these findings, evaluation of biological-fluid extracts as P-gp inhibitors was carried out by measuring their effects on the transepithelial transport of Rho123 across Caco-2 cell monolayers or the accumulation of [³H]DNR in LLC-GA5-COL150 cells.

**Inhibitory potency of human urine extract on P-gp function:** Effect of hydrophobic human urine extract, collected from a 23-years old male subject, on b-to-a transport of Rho123 across Caco-2 cell monolayers was examined. Urine extract was added in the transport medium at different final concentrations as follows: ×2.5, 2.5-fold higher concentration of original urine (extract from 2.5-mL human urine/mL of transport medium); ×5, concentrated by 5-fold; ×10, concentrated by 10-fold. With ×2.5 urine extract, the b-to-a transport of Rho123 was not affected, but with ×5 and ×10 urine extracts, the b-to-a transport of Rho123 was significantly suppressed (data not shown). With ×5 urine extract, 6 samples out of 7, collected on different days (during from December 3rd, 1997 to August 4th, 1998) from the same male subject, suppressed the P-gp-mediated efflux transport of Rho123 in Caco-2 cells (Fig. 1). These results indicate that human urine con-
Inhibitory potency of human urine extract, collected from a 23-year old male subject on different days (December 3rd, 1997–August 4th, 1998), on the basal-to-apical transport of Rho123 across Caco-2 cell monolayers. Rho123 (5 μM) was placed at the basal side of monolayers, and human urine extract was added in the transport medium at a 5-fold higher concentration of original urine. The transport study was carried out for 120 min in the absence (control) or presence of urine extract. Sampling date for each sample number: 1, 12/3 (1997); 2, 2/6 (1998); 3, 6/3 (1998); 4, 6/4 (1998); 5, 7/7 (1998); 6, 7/25 (1998); 7, 8/4 (1998). Each value is the mean ± S.E. of 3 trials. The value without error bar is the result of 1 trial.

Similarly, urine extracts were prepared from 15 different individuals (8 males, 7 females), and their inhibitory potencies on P-gp function were compared using ×5 extract (Fig. 2A and B). Their inhibitory potencies varied remarkably among different individuals, and 11 samples out of 15 inhibited the P-gp function. Among them, urine extract of a female (39 years old), obtained 1 month after delivery, showed a greater inhibitory potency than other extracts, and urine extracts of 3 and 5-years old girls showed no inhibitory effects (Fig. 2B).

In a separate experiment, urine from a pregnant female 1 week before delivery was examined in the accumulation study of [3H]DNR in LLC-GA5-COL150 cells. The accumulation of [3H]DNR in cells increased by approximately 1.8-fold of control at the same concentration as the original urine (×1 extract) (Fig. 3). These results may suggest that urine from a pregnant female has much greater inhibitory effect on P-gp function than other extracts.

Inhibitory potencies of rat urine and plasma extracts on P-gp function: Urine and plasma extracts were prepared by ethylacetate extraction from normal, glycerol-induced ARF and CCl4-induced AHF rats, and their effects on transport of Rho123 across Caco-2 cell monolayers were examined. As compared with human urine extract, urine extract from normal rats showed P-gp inhibitory effect even at a very low concentration (×0.0125, 80-fold dilution). The inhibitory potencies of
Fig. 4. Effect of rat urine (A) and plasma (B) extracts obtained from normal (●), glycerol-induced acute renal failure (■), and CCl₄-induced acute hepatic failure (▲) rats on the basal-to-apical transepithelial transport of Rho123 across Caco-2 cell monolayers. The concentration of Rho123 was 5 μM. Transport study was carried out for 120 min in the absence (○) or presence (●, ■, ▲) of urine (×0.0125, A) or plasma (×0.025, B) extract. Each value is the mean ± S.E. of 2–12 trials.

Fig. 5. Separation of human urine extract collected from 23-years old male subjects by a two-dimensional TLC. Solid phase was silica gel GF₂₅₄. Detection was made with UV lamp.

Fig. 6. Inhibitory effect of each spot fraction on basal-to-apical transepithelial transport of Rho123 across Caco-2 cells. Spot fraction: human urine extract obtained from 100-mL urine of 23-years old male subject was separated by a two-dimensional TLC (see Fig. 5 for each spot number). Rho123 (5 μM) was placed at the basal side of monolayers, and each spot fraction was applied in the transport medium. Transport study was carried out for 120 min in the absence (control) or presence of a spot fraction. Each value is the mean ± S.E. of 2 trials, except fraction No. 10 (1 trial).

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urine and plasma extracts varied depending on their physiological states, in which ARF rat urine extract showed no inhibitory effect and the inhibitory potency of AHF rat urine extract was lower than that of normal rats (Fig. 4A). In contrast to those of urine extracts, the inhibitory potencies of plasma extracts were in reverse order as follows: control (without plasma extract) = normal < AHF < ARF rat plasma (Fig. 4B). These results indicate that P-gp inhibitors are normally excreted into the urine, but they are accumulated in the plasma under diseased states, especially under ARF states.

Separation of human urine extract by a two-dimensional TLC and inhibitory potencies of the components on P-gp function: Human urine was collected from a 23-years old male subject (same person as for Figs. 1 and 2) for several days and combined together. Urine extract was prepared by extracting with ethylacetate, and the extract obtained from 100-mL urine was applied on a 20 × 20-cm TLC plate to separate their components (Fig. 5). Some spot fractions were scraped off to collect urine components, and their inhibitory potencies on the b-to-a transport of Rho123 were examined in Caco-2 cells (Fig. 6). Although the recovered amounts of urine components were different among different spot fractions, some fractions (spot No. 1, 2, 6–9, 11) showed inhibitory effects on P-gp function.

Isolation and identification of endogenous P-gp inhibitor(s): Among various spots shown in Fig. 5, the fraction of spot No. 8 had a relatively large amount of components. Analysis of No. 8 fraction by GC-MS indicated that this spot contains more than 10 different components and equilin was found within them. Identification was made by comparing with GC-MS spectrum of standard equilin. As well, 3α-hydroxy-5β-androsten-11,17-dion, stearic acid, and adipic acid were found in No. 8 fraction. Since the effect of equilin on P-gp function has not yet been reported, the inhibitory effect of equilin, together with estriol, estron, and 17β-estradiol (a standard of estrogen), on [3H]DNR accumulation in LLC-GA5-COL150 cells was examined. At a concentration of 100 μM, equilin and estriol, as well as 17β-estradiol, increased the accumulation of [3H]DNR significantly (Fig. 7).

Discussion

The inhibitory potencies of hydrophobic extracts pre-
These include aldosterone, corticosterone, cortisol, testosterone, and so on. Among these steroid hormones, cortisol and progesterone are reported to be the greatest inhibition. Also, the concentration of corticosterone in the plasma was approximately 2-fold higher in these diseased rats, as compared with normal rats. However, similar cases are sometimes observed, and some P-gp substrates were or were not transported in a polarized mode, depending on cell types expressing P-gp.

In evaluating P-gp-inhibitory effect of biological-fluid extracts, a sensitive evaluation system is prerequisite. For example, in Caco-2 cells, the inhibitory effect of verapamil on the efflux (b-to-a) transport of Rho123 was clearly observed, however, the enhancing effect of verapamil on a-to-b transport of Rho123 was small. Also, the effect of verapamil on the accumulation of Rho123 in Caco-2 cells was small (data not shown). These results indicate that the inhibitory effect of verapamil on P-gp function could be sensitively detected by examining the effect on Rho123 efflux transport, rather than the effect on a-to-b transport or accumulation of Rho123 in Caco-2 cells. In contrast to Caco-2 cells, efflux transport and accumulation of [3H]DNR were found to be more sensitive parameters than those of Rho123, when LLC-GA5-COL150 cells were used. The reason for such differences between cells is not clear at present.

In human urine study, the sampling time of urine, the intake amount of water, and meals were not controlled nor restricted, although the intake of P-gp-related drugs was restricted. Therefore, the apparent intra- and inter-individual variations of P-gp inhibitory potencies of human urine extracts observed in the present study may partly be derived from such experimental protocols. With ×5 human urine extracts, more than half samples derived from the same or different individuals showed inhibitory effects on in-vitro P-gp function (Figs. 1 and 2). Among these samples, urine obtained from little girls (3 and 6 years old) showed no inhibitory effect and a female urine, 1 month after delivery, exhibited the greatest inhibition (Fig. 2B). Also, in accumulation study of [3H]DNR in LLC-GA5-COL150 cells, the urine from a pregnant female showed P-gp-inhibitory effect even at the same concentration of its original urine (Fig. 3). The greater inhibitory potency of female urine collected before and after delivery may be due to the higher levels of steroid hormones such as estriol and pregnanediol in the urine. Although further study is necessary to clarify the reasons for the different P-gp inhibitory potencies among different individuals, the physiological states of hosts such as age, pregnancy and diseases would relate to the P-gp-inhibitory potencies of their urine extracts.

Rat urine extract, as well as rat plasma extract, was analyzed mainly in terms of the effect of disease states. We have already reported the inhibitory effect of ARF and AHF rat plasma on P-gp function, although we used the plasma itself without extraction in those studies. Also, it has been found that the plasma filtrate of AHF rats, obtained by ultrafiltration using semipermeable cellulose membrane, showed no inhibitory effect.

These results suggested that endogenous P-gp inhibitors are lipophilic and mostly bound to plasma.
proteins, as reported elsewhere. Interestingly, among different physiological states, the rank order of inhibitory potencies was in reverse order between urine and plasma extracts as follows: urine extracts, control (without urine extract) = ARF < AHF < normal rat urine extracts; plasma extracts, control (without plasma extract) = normal < AHF < ARF rat plasma extracts (Fig. 4). These results indicate that endogenous P-gp inhibitors are excreted into the urine under normal conditions, but they are accumulated in the plasma under ARF and AHF states.

Some spot fractions of human urine extract separated by a two-dimensional TLC showed P-gp-inhibitory effects (Figs. 5 and 6). The different inhibitory potencies among different spot fractions would depend on the difference in its amount and/or composition of each fraction. Fractions of spot No. 1 and 2 showed relatively strong inhibitory potencies as compared with other fractions (Fig. 6). However, they are fairly hydrophilic among components in ethylacetate extract as evaluated from their Rf values (Fig. 5). It is generally accepted that the substrates and/or inhibitors of P-gp are hydrophobic, uncharged or positively charged compounds. Therefore, in the present study, more hydrophobic fractions were examined further. The amounts recovered from spot No. 8, 10 and 11 were relatively large, and No. 8 fraction contained more than 10 components when separated by GC-MS. Also, No. 11 fraction was further separated into more than 8 components by HPLC (data not shown). In No. 8 fraction, equilin was detected, and equilin, as well as estriol, was newly found to be one of endogenous P-gp inhibitors (Fig. 7). Further study is necessary to isolate and identify other endogenous P-gp inhibitors/substrates in human urine.

In the present study, we examined the P-gp-inhibitory potencies of human and rat urine extracts. Urine is the fluid that escaped from tubular reabsorption and about 99% of water filtered through glomerulus is normally reabsorbed. Thus, concentrations of endogenous P-gp inhibitors in the fluid at proximal renal tubules, where P-gp is expressed, may be much lower than those in the urine, although the extent of reabsorption of endogenous P-gp inhibitors is unclear. When considering the inhibitory potency of urine extract and experimentally employed concentration of extract in the medium, even urine from a pregnant female may not directly inhibit P-gp function at the proximal renal tubules. However, disease states such as kidney dysfunction may markedly increase the risk of systemic P-gp suppression, because the basal levels of P-gp inhibitors in the biological fluids would be very high during pregnancy. On the other hand, rat urine extract showed significant P-gp-inhibitory effect even at diluted concentrations. It has been reported that the effective concentration of the substance in rat urine required to inhibit in-vitro P-gp function was similar and within the range of its urinary concentration. However, our results indicate that P-gp-inhibitory potency of rat urine is greater and it may interfere with P-gp even at proximal renal tubules. The difference of P-gp inhibitory potencies between human and rat urine extracts may be clarified by comparing urine components as well as their concentrations. Also, such investigation may lead to the development of endogenous, safer and potent multidrug resistance-reversing agents.

In conclusion, it was demonstrated that urine contains many endogenous P-gp inhibitors, and their inhibitory potencies on P-gp function vary with intra- and inter-individual variations, and under different physiological states.

Acknowledgement: This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and by a grant from the Naito Foundation, Japan.

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