Laboratory of Biopharmaceutics and Pharmacokinetics[1], Faculty of Pharmaceutical Sciences, Hiroshima International University; Department of Pathophysiology and Therapeutics[2], Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

Interaction of hydrophobic components in female urine before and after childbirth with P-glycoprotein *in vitro*

T. Yokooji[1,2], Y. Kameda[1], M. Utsumi[1], N. Mori[1], T. Murakami[1]

Received November 4, 2013, accepted November 29, 2013

Teruo Murakami, Laboratory of Biopharmaceutics and Pharmacokinetics, Faculty of Pharmaceutical Sciences, Hiroshima International University, 5-1-1 Hiro-koshingai, Kure, Hiroshima 737-0112, Japan
t-muraka@ps.hirokoku-u.ac.jp

Pharmazie 69: 430–436 (2014) *doi: 10.1691/ph.2014.3907R*

The first urine in the morning (total 15 samples) and whole day urine (total 4 days, 17 samples) were collected from a young healthy woman during the pregnancy and lactation period, to examine the possible interactions of urine components (methanol extracts) with P-glycoprotein (P-gp) and multidrug resistance-associated proteins (MRPs). The interaction was evaluated by measuring the intracellular accumulation of rhodamine123, a P-gp substrate, in LLC-GA5-COL150 cells, or calcein, an MRP substrate, in Caco-2 cells in the absence and presence of urine components. Four first urine samples out of 12 collected before childbirth and one sample out of three collected after childbirth suppressed P-gp function significantly. The effect of pregnancy and lactation on P-gp inhibitory potencies of urine components was not observed. The whole day urine samples showed a clear circadian rhythm, in which three first urine samples in the morning out of four showed greater P-gp inhibitory potencies than other daytime samples. Interaction of urine components with MRPs was not detected. In conclusion, the concentration of endogenous P-gp inhibitor(s) was higher in the first urine in the morning, showing a clear circadian rhythm. Normal pregnancy and lactation appeared not to significantly affect the P-gp inhibitory potencies of urine components.

1. Introduction

ATP-dependent efflux transporters such as P-glycoprotein (P-gp) and multidrug resistance-associated proteins (MRPs) are expressed in various normal tissues including the intestine, liver, kidney, brain and placenta (Kool et al. 1997; Takano et al. 2006). These ATP-binding cassette (ABC) transporters have important roles in preventing the intracellular accumulation of endogenous and exogenous substrate compounds by limiting the influx into cells and facilitating the efflux out of cells. P-gp transports relatively hydrophobic compounds such as steroidal hormones, immunosuppressants, calcium channel blockers, anticancer agents and anti-human immunodeficiency virus agents. In contrast, MRPs transport relatively hydrophilic compounds such as methotrexate, pravastatin and various conjugated compounds. Like this, both P-gp and MRPs also recognize exogenously administered compounds, in addition to endogenously produced compounds such as estrogens, corticosterone, progesterone, and bilirubin glucuronides (Ueda et al. 1992; Rao et al. 1994; Orlowski et al. 1996; Takano et al. 2006; Yokooji et al. 2010, 2011). Biological fluids such as plasma and urine contain various endogenous P-gp and MRP substrate/modulators (Ichikawa et al. 1990; Ichikawa-Haraguchi et al. 1993; Charuk and Reithmeier 1992; Charuk et al. 1994; Rao et al. 1994; Mülder et al. 1996; Murakami et al. 2002a, 2002b; Mutsaers et al. 2011), though the inhibitory potencies of biological fluids against the transporter-functions could vary depending on the physiological states of hosts (Huang et al. 2000, 2001; Murakami et al. 2002a, 2002b; Yumoto et al. 2003; Yokooji et al. 2006, 2011). It is known that, in the late stage of pregnancy, the plasma and urinary levels of steroidal hormones such as estrogens and cortisol, which are known as endogenous modulators for P-gp and MRPs, are increased by approximately 100- and 1000-fold of non-pregnant states, respectively, with great circadian variations (Adlercreutz et al. 1967; Burke and Roulet 1970; Rado et al. 1970; Tulchinsky et al. 1972; Townsley et al. 1973; Ueda et al. 1992; Rao et al. 1994). Based on such information, in the present study, urine was collected from a young healthy woman before and after childbirth, hydrophobic urine components were extracted by methanol using a reversed phase cartridge, and the inhibitory potencies of urine extracts on P-gp and MRP functions were examined *in vitro*. Inhibitory potencies against P-gp and MRPs were evaluated by measuring the intracellular accumulation of rhodamine123 (Rho123), a P-gp substrate, in LLC-GA5-COL150 cells, and calcein, an MRP substrate, after application of calcein acetoxymethyl ester (calcein-AM) in Caco-2 cells, respectively. LLC-GA5-COL150 cell is a porcine kidney epithelial cell line (LLC-PK_1) transfected with human MDR1 cDNA, which overexpresses human P-gp on the apical membrane (Ueda et al. 1992; Tanigawara et al. 1992). Caco-2 cell, a human colonic adenocarcinoma cell line, expresses various ABC and solute carrier (SLC) transporters including P-gp and MRPs, as well as human intestine (Hunter et al. 1993; Wils et al. 1994; Prime-Chapman et al. 2004; Englund et al. 2006; Seithel et al. 2006).

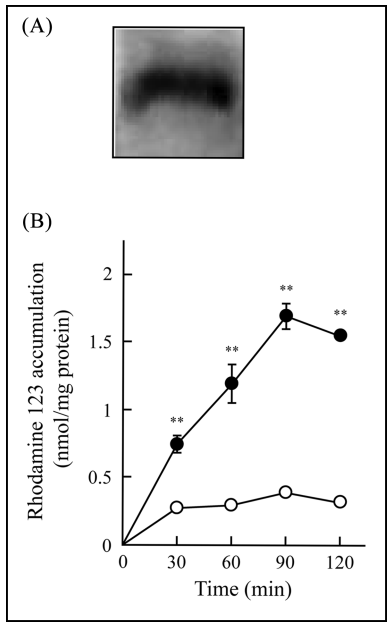

Fig. 1: (A) Western blot analysis of P-glycoprotein in crude membranes from LLC-GA5-COL150 cells. Crude membrane fractions (20 μg protein) were subjected to SDS-PAGE. The proteins were blotted on a PVDF membrane and detected with a primary antibody for P-gp (C219). (B) Effect of verapamil on the accumulation of rhodamine 123 in LLC-GA5-COL150 cells. The accumulation of rhodamine 123 (20 μM) in the absence (opened circles) or presence (closed circles) of 300 μM verapamil was measured at 37°C after 30, 60, 90, and 120 min-incubation. Each value represents the mean ± S.E.M. of three trials. $^{**}P<0.01$, $^{*}P<0.05$: significantly different from the value in the absence of verapamil.

2. Investigations and results

2.1. Expression and function of P-gp in LLC-GA5-COL150 cells

Expression of P-gp in LLC-GA5-COL150 cells was evaluated by Western blot analysis, and band of approximately 170 kD corresponding to the molecular size of P-gp was detected in the CM fraction of LLC-GA5-COL150 cells (Fig. 1A). The functional expression of P-gp was also examined by comparing the intracellular accumulation of Rho123 in the absence (control) and presence of verapamil, and the intracellular accumulation of Rho123 was significantly increased by approximately 4.3-fold of control in the presence of verapamil (Fig. 1B).

2.2. Interaction of hydrophobic urine extracts with P-gp

The interaction of hydrophobic urine extracts from a pregnant and lactating woman with P-gp was examined by measuring the intracellular accumulation of Rho123 (30 min incubation) in LLC-GA5-COL150 cells. Four urine samples out of 12 collected before childbirth and one sample out of three collected after childbirth significantly increased Rho123 accumulation (Fig. 2). In the case of whole day urine samples, the inhibitory potencies of urine extracts varied remarkably depending on the sampling times, and urine extracts prepared from the first urine in the morning showed greater inhibitory potencies on P-gp function than other urine extracts, and the potency decreased with time along the day time (Fig. 3).

2.3. Expression of ABC transporters and interaction of urine extracts with MRPs in Caco-2 cells

Expression of P-gp, MRP2 and MRP3 in Caco-2 cells was evaluated by Western blot analysis, and bands of approximately 170 kD corresponding to the molecular size of P-gp and approximately 190 kD corresponding to the molecular sizes of MRP2 and MRP3 were observed in the CM fraction of Caco-2 cells (Fig. 4A). Calcein, a hydrolysate of calcein-AM, is known as a specific substrate for MRPs and calcein-AM is known as a substrate for P-gp (Essodaigui et al. 1998; Evers et al. 2000). The effects of MK-571, an MRP inhibitor, and verapamil, a P-gp inhibitor, on the intracellular accumulation of calcein after application of calcein-AM were also examined to elucidate the functional expression of MRPs and P-gp in Caco-2 cells (Fig. 4B). Both MK-571 and verapamil significantly increased the intracellular accumulation of calcein by approximately 3-fold and 2.5-fold that of calcein-AM alone, possibly due to the inhibition of MRPs- and P-gp-mediated efflux of calcein and calcein-AM, respectively. The mixture of MK-571 and verapamil further increased the accumulation of calcein by approximately 5-fold that of control.

Based on these findings, the interaction between urine extracts and MRPs was examined in the presence of verapamil, to avoid the P-gp-mediated efflux of calcein-AM. However, the calcein accumulation was not affected by the presence of urine extracts, in all urine extract samples examined (Figs. 5 and Fig. 6).

3. Discussion

Biological fluids such as plasma and urine contain various endogenous P-gp and MRP modulators in human and rats (Ichikawa et al. 1990; Charuk and Reithmeier 1992; Charuk et al. 1994; Rao et al. 1994; Mülder et al. 1996; Huang et al. 2000, 2001; Murakami et al. 2002a, 2002b; Yumoto et al. 2003; Yokooji et al. 2011). Murakami et al. (2002a) reported that the plasma samples obtained from glycerol-induced acute renal failure (ARF) and carbon-tetrachloride-induced acute hepatic failure (AHF) rats exhibited potent P-gp inhibitory effects, in which higher plasma concentrations of corticosterone, an endogenous P-gp substrate/inhibitor, were observed. Yumoto et al. (2003) reported that the *in-vivo* intestinal P-gp function was significantly lower in AHF rats, though the expression of P-gp and *in-vitro* P-gp function were significantly higher in AHF rats, as compared with those in untreated control rats. The discrepancy between the *in-vitro* and *in-vivo* P-gp functions in AHF rats was considered to be due to the accumulation of endogenous modulators in the body of AHF rats. Recently, we also demonstrated that hyperbilirubinemia accompanied by obstructive jaundice can increase harmful toxicities of exogenously administered Mrps and/or Oatps substrate drugs at various tissues, by suppressing the efflux transporter's function systemically in rats model (Yokooji et al. 2010, 2011).

The levels of steroidal hormones such as cortisol, progesterone and estrogens in blood and urine are known to be higher during pregnancy with wide circadian variation in a day, compared to those in non-pregnancy (Adlercreutz et al. 1967; Burke et al. 1970; Rado et al. 1970; Tulchinsky et al. 1972; Townsley et al. 1973). The higher steroidal hormone levels in the body during pregnancy may suppress the function of transporters and modulate the pharmacokinetics of substrates for transporters, since many steroidal compounds and their metabolites are substrates of P-gp and/or MRPs (Ueda et al. 1992; Rao et al. 1994; Yumoto et al. 2003; Takano et al. 2006; Murakami and Takano 2008). In relation to pregnancy, Hebert et al. (2008) reported that the hepatic and/or intestinal CYP3A and renal P-gp activities were increased during pregnancy in human. Zhang et al. (2009) reported that plasma concentrations of protease inhibitors were lower in pregnant women than those in non-pregnant women or men, and in a representative non-human primate model, *Macaca nemestrina*. They proposed that factors other than increased CYP3A or P-gp activity contribute to the increased clearance of

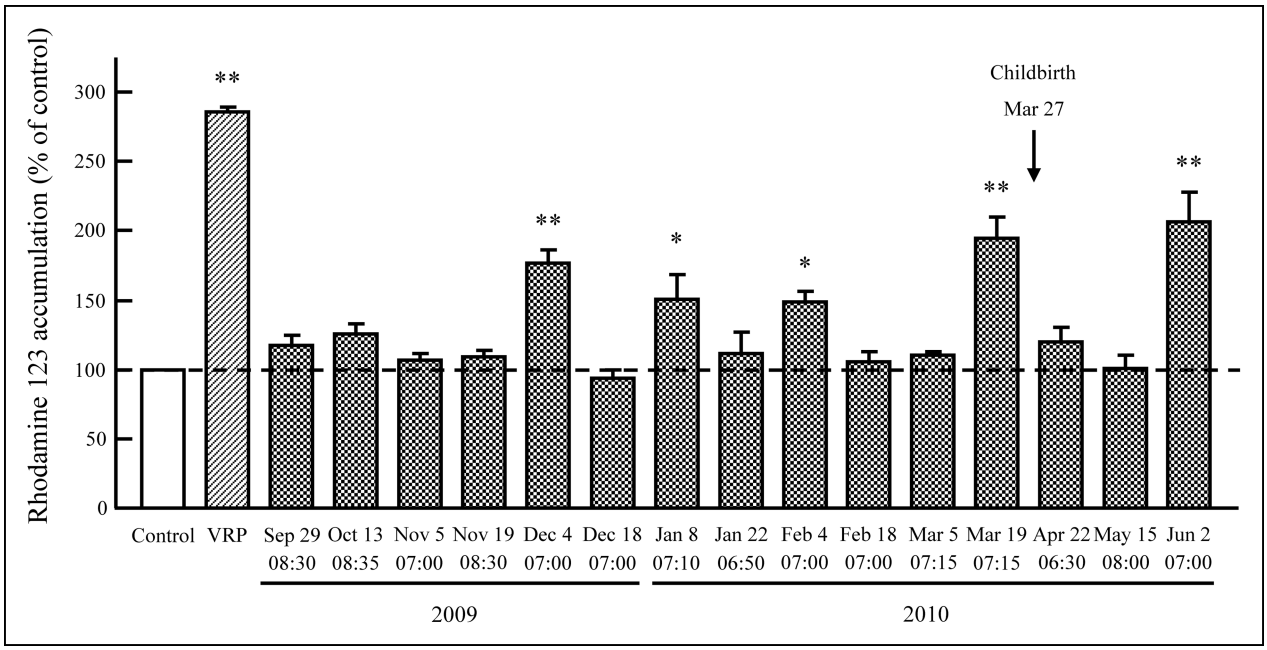

Fig. 2: Effect of human urine extracts prepared from female urine, collected at first morning on different days during pregnancy and postpartum, on the accumulation of rhodamine 123 in LLC-GA5-COL150 cells. The cellular accumulation of Rho123 (20 μM) in the absence (control) or presence of verapamil (VRP, 300 μM) or urine extracts was measured after 30 min-incubation at 37°C. Human urine extract was added to the uptake medium at the same concentration with that in original urine. Each value represents the mean ± S.E.M. of three trials. $^{**}P < 0.01$: significantly different from the value for control.

protease inhibitors during *M. nemestrina* pregnancy. However, the detailed mechanism underlying the effect of pregnancy on pharmacokinetics of P-gp substrates is not yet fully understood. In the present study, we evaluated possible interactions of hydrophobic urine components of a pregnant and lactating woman with P-gp and MRPs. The interaction of urine extracts with P-gp was evaluated by measuring the intracellular accumulation of Rho123 in LLC-GA5-COL150 cells. The interaction with MRPs was evaluated by measuring the accumulation of calcein after application of calcein-AM in Caco-2 cells in the absence and presence of urine extracts. In this latter study, verapamil was also added, in addition to urine extracts, to prevent the efflux of calcein-AM, since calcein-AM is a substrate of P-gp (Essodaigui et al. 1998; Evers et al. 2000) (Fig. 4). Four urine samples out of 12 collected before childbirth and one sample out of three collected after childbirth suppressed P-gp function significantly (Fig. 1). However such inhibitory potencies of urine components have also been observed even with non-pregnant female and male urine samples (Murakami et al. 2002b). A rational relationship was not observed between the timing of childbirth and the P-gp inhibitory potencies of urine extracts. In addition, urine extracts collected 3 month after childbirth

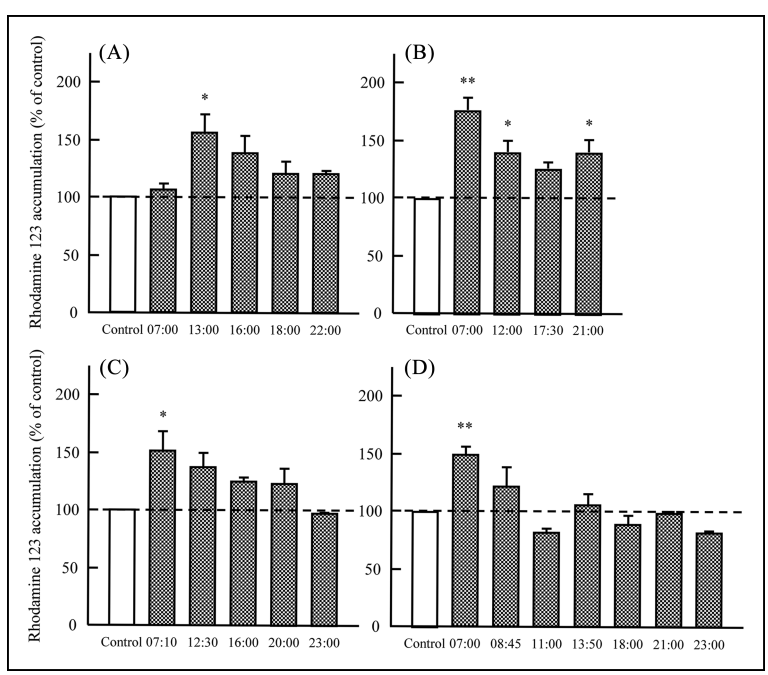

Fig. 3: Effect of human urine extracts prepared from female urine, collected at different times in each day during pregnancy and postpartum, on the accumulation of rhodamine 123 in LLC-GA5-COL150 cells. Urine was collected at different times in 11/5 (2009) (A), 12/4 (2009) (B), 1/8 (2010) (C) and 2/4 (2010) (D). Cellular accumulation of Rho123 (20 μM) in the absence (control) or presence of verapamil (VRP, 300 μM) or urine extracts was measured after 30 min-incubation at 37°C. Human urine extract was added to the uptake medium at the same concentration with that in original urine. Each value represents the mean ± S.E.M. of three trials. $^{**}P < 0.01$, $^{*}P < 0.05$: significantly different from the value for control.

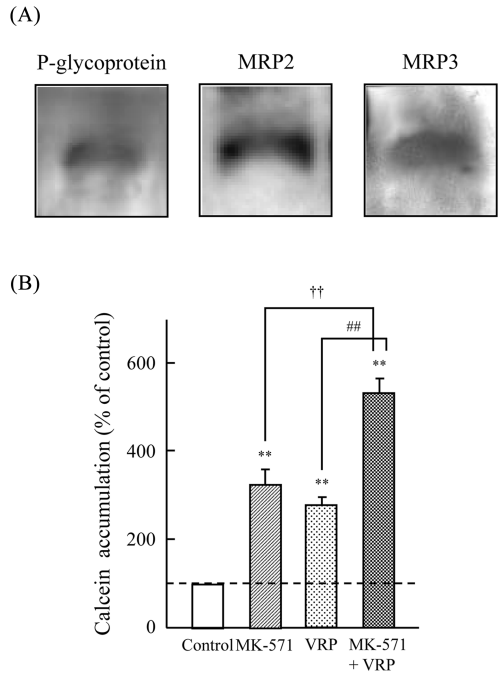

Fig. 4: (A) Western blot analysis of P-gp, MRP2 and MRP3 in crude membranes of Caco-2 cells. Crude membrane fractions (20 μg protein) were subjected to SDS-PAGE. The proteins were blotted on a PVDF membrane and detected with a primary antibody for MRP2 (M_2III-6) and MRP3 (D-15). (B) Effect of MK-571 and verapamil (VRP) on the accumulation of calcein in Caco-2 cells. The accumulation of calcein after application of calcein-AM (2 μM) in the absence or presence of MK-571 (25 μM) and/or verapamil (300 μM) was measured after 30 min-incubation at 37°C. Each value represents the mean ± S.E.M. of three trials. $^{**}P < 0.01$: significantly different from the value for control. $^{††}P < 0.01$: significantly different from the value for MK-571. $^{\#\#}P < 0.01$: significantly different from the value for verapamil.

showed a significantly greater P-gp-inhibitory potency, though 1 and 2 month urine samples after childbirth showed no P-gp inhibitory potencies. It is reported that the plasma estrogen levels 3 months after childbirth are similar to those under non-pregnant normal conditions (Rado et al. 1970). Thus, it was considered that normal pregnancy and lactation of healthy pregnant women does not cause interaction between plasma components in central circulation and P-gp expressed in the body, as well as those in non-pregnant women and men.

We also examined the effects of sampling time of urine on P-gp inhibitory potencies. Three first urine samples in the morning out of four showed greater P-gp inhibitory potencies than other day time samples, and the P-gp inhibitory potencies decreased with time towards the evening (Fig. 3). The circadian rhythmus of steroidal hormone levels, such as the concentrations of cortisol and dehydroepiandrosterone (DHEA) in biological fluids, are well and widely recognized. For example, it is reported that the circadian rhythm of serum cortisol, a potent P-gp substrate/inhibitor, was characterized by peaks (04:00–06:00) and troughs (18:00–24:00) in healthy Chinese men, occurring approximately 2 h earlier than those usually reported in Caucasians (Zhao et al. 2003). The circadian rhythmus of steroidal hormones in maternal plasma during pregnancy is also well recognized. For example, cortisol, estrone, and estriol in maternal venous plasma showed a clear circadian rhythmus and the maternal cortisol was maximal at 07:30 h and fell significantly to its lowest concentration at 02:30 h. The level of maternal estriol was lowest between 10:00 and 11:30 h and rose significantly to maximal values between 22:30 and 00:30 h, and the level of maternal estrone was maximal between 10:00 and 11:30 h and showed a significant decrease to reach its lowest concentration at 04:30 to 06:30 h. In contrast, no significant circadian change was observed in maternal estradiol levels (Patrick et al. 1979; Patrick et al. 1980). The effect of gestational age is also reported, in which the circadian changes in the levels of estrone were apparent at 34 to 35 and 38 to 39 weeks of pregnancy, and peak values were measured around 08:30 to 09:30 h and troughs occurred between 01:30 and 05:30 h. In addition, nighttime concentrations of estradiol were lower than the peak values at 07:30 to 08:30 h (Challis et al. 1980; Challis et al. 1983). The greater P-gp inhibitory potencies of first urine extracts in the morning would not be explained by only one steroidal hormone level, but the circadian pattern of P-gp inhibitory potencies of urine extracts observed in the present study appeared to be corresponding to the circadian rhythmus of some steroidal hormones such as cortisol in central circulation.

The interaction between urine extracts and MRPs was not detected in the present study (Figs. 4–6). It is generally accepted that the substrates for MRPs are hydrophilic, uncharged or negatively charged compounds. It is considered that many MRP substrates, such as conjugated compounds of steroidal hormones, will also be excreted into urine during pregnancy. However, in this study, the interaction with MRPs was evaluated by using methanolic urine extracts. Thus, it is unclear whether endogenous substrates for MRPs in urine were extracted or not by the present extraction method, because MRP substrates are mostly hydrophilic compounds. In addition, it is speculated that hydrophilic MRP substrates are low membrane permeability, and therefore the interaction with MRPs could be low, if any. Further study is necessary to evaluate the plausible interaction of urine components of pregnant and lactating woman with MRPs.

In conclusion, in the present study, we examined the possible interaction of hydrophobic urine components obtained from a pregnant and lactating woman with P-gp and MRPs, since the urine of pregnant woman can contain various endogenous P-gp substrates/inhibitor(s) at higher concentrations, as compared with those in non-pregnant women. Some urine samples showed significant inhibitory potencies on P-gp function, but not on MRPs function. However, P-gp inhibitory potencies of urine extracts have been also observed in non-pregnant female and male urine (Murakami et al. 2002b), and no rational relationship was observed between the timing of childbirth and P-gp inhibitory potencies of urine extracts. In contrast, a fairly clear circadian rhythm was observed in the P-gp inhibitory potencies of urine extracts, in which P-gp inhibitor(s) was greater in the first urine in the morning. Assuming that the urine components are concentrated by approximately 100 folds as compared with that in plasma, the interaction between steroidal hormones in systemic circulation of healthy pregnant woman and P-gp expressed in the body would be negligible or very small extent, if any.

4. Experimental

4.1. Materials

Rho123 was obtained from Acros Organics (Geel, Belgium). Verapamil hydrochloride, a typical P-gp inhibitor, calcein, a typical MRP substrate, and calcein-AM (acetoxymethyl ester of calcein) were purchased from Wako Pure Chemicals (Osaka, Japan). MK-571, a specific MRP inhibitor, was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). C18 solid phase extraction cartridges, Bond Elut®, were from Varian (Palo Alto, CA, USA). All other chemicals used were of the highest purity available.

4.2. Extraction of methanolic hydrophobic components in urine

Urine was collected at intervals from a 23-year-old healthy woman before and after childbirth during September 29, 2009 and June 2, 2010. First urine in the morning was collected 15 days in total. Also, in four days out of 15, daytime urine was also collected serially, and the sampling times of urine were recorded. During these days throughout the urine collection, she took neither the liquor nor the cigarette nor the medicine. Agreement of using

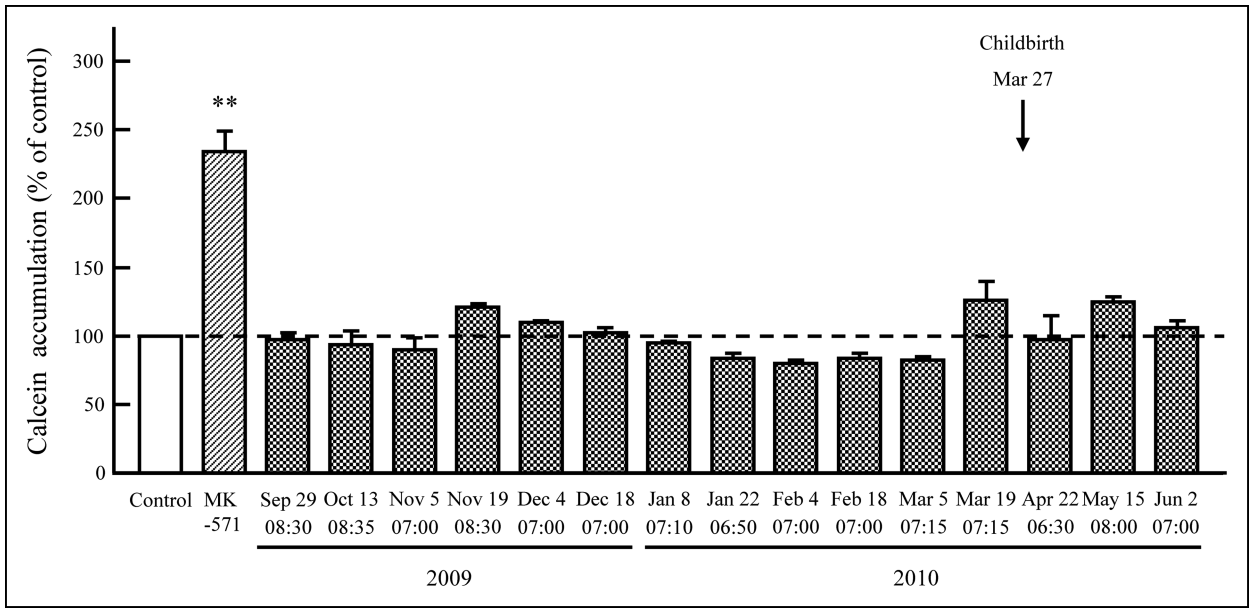

Fig. 5: Effect of human urine extracts prepared from female urine, collected at first morning on different days during pregnancy and postpartum, on the accumulation of calcein in Caco-2 cells. Cellular accumulation of calcein after application of calcein-AM (2 μM) in the absence (control) or presence of verapamil (VRP, 300 μM) or urine extracts was measured after 30 min-incubation at 37°C. Human urine extract was added to the uptake medium at the same concentration with that in original urine. Each value represents the mean ± S.E.M. of three trials. Significant effect of urine extracts was not observed.

her urine for interaction study with ABC transporters was obtained with a written informed consent. The urine sample collected at intervals was injected at a volume of 4 ml into Bond Elut C18, a reversed phase cartridge, and then 10 ml of distilled water was injected to remove hydrophilic compounds. The retained compounds in the cartridge were eluted with 2 ml of methanol, and the solvent was evaporated to dryness under reduced pressure. The residue, methanolic hydrophobic urine extracts, was stored at −30 °C until use. In inhibition studies using urine extracts, the residue obtained from 4 ml urine was dissolved in 4 ml of pH 7.4 phosphate-buffered saline (PBS: 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2HPO_4, 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$, 5 mM D-glucose) containing 1% dimethylsulfoxide (DMSO) to reconstruct the same concentrations of urine components as that in original urine.

4.3. Cell culture

LLC-GA5-COL150 cells (Riken Cell Bank, Ibaraki, Japan) between passages 6 and 10, and Caco-2 cells between passages 70 and 77 were cultured in the same manner as described previously (Tanigawara et al. 1992; Ueda et al. 1992; Takano et al. 1998; Yumoto et al. 1999). Briefly, LLC-GA5-COL150 cells and Caco-2 cells were seeded at a density of 10×10^5 cells on a 100 mm-dish and cultured with culture medium for 7 days to analysis transporter expression. For inhibition studies using urine extracts, cells were seeded at a density of 5×10^4 cells/well on the 24-well plates and cultured for 14 days. Fresh medium was replaced every 2 days. In case of LLC-GA5-COL150 cells, colchicine was removed from the culture medium one day before the accumulation study, in the similar manner as reported previously (Murakami et al. 2002b).

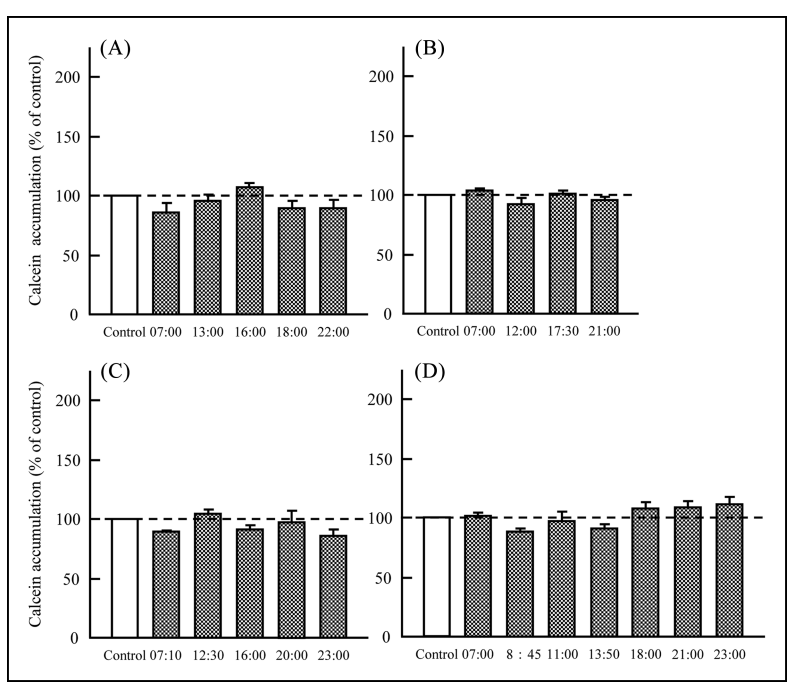

Fig. 6: Effect of human urine extracts prepared from female urine, collected at different times in each day during pregnancy and postpartum, on the accumulation of calcein in Caco-2 cells. Urine was collected at different times on 11/5 (2009) (A), 12/4 (2009) (B), 1/8 (2010) (C) and 2/4 (2010) (D). Cellular accumulation of calcein after application of calcein-AM (2 μM) in the absence (control) or presence of verapamil (VRP, 300 μM) or urine extracts was measured after 30 min-incubation at 37°C. Human urine extract was added to the uptake medium at the same concentration with that in original urine. Each value represents the mean ± S.E.M. of three trials. Significant effect of urine extracts was not observed.

4.4. Western blot analysis

The expressions of P-gp in LLC-GA5-COL150 cells and P-gp, MRP2 and MRP3 in Caco-2 cells were evaluated by Western blot analysis after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using crude cell membrane (CM) in the same manner as reported previously, with a slight modification (Yokooji et al. 2007; Patanasethanont et al. 2007a, 2007b). Briefly, the culture medium was removed and each dish was washed with ice-cold D-PBS (pH 7.4). Tris-HCl buffer (pH 7.5: 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM Tris) was added, and the cells were collected with rubber policeman. The cell suspension was homogenized for 2 min with a Polytron tissue homogenizer (IKA, Labortechnik, Staufen, Germany) and was subsequently homogenized by means of a glass-Teflon Potter homogenizer (1,000 rpm, 10 strokes). The homogenate was centrifuged at 3,000 × g for 10 min and then the supernatant was centrifuged at 105,000 × g for 65 min. The pellet was resuspended in the ice-cold buffer containing 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 10 mM Tris, 0.1% SDS and 1% deoxycholic acid and centrifuged at 14,000 × g for 15 min. The supernatant containing the CM fractions was then adjusted at 5 mg/ml of protein. All procedures were performed on ice or at 4 °C, and the suspended samples were used immediately after preparation or stored in liquid nitrogen until use. Protein concentration in CM preparations was measured by the Lowry method using bovine serum albumin as the standard (Lowry et al. 1951). The CM samples were applied to a gel at an amount of 20 μg proteins, and SDS-PAGE was performed. For the detection of P-gp and MRP2, C219 (1:10) and M_2III-6 (1:50 dilution) were used as the primary monoclonal antibodies, respectively. As the secondary antibody, peroxidase-labeled affinity-purified antibody to mouse IgG (1:1000 dilution) was used. For the detection of MRP3 protein, D-15 (1:50 dilution) and peroxidase-labeled affinity-purified antibody to goat IgG (1:1000 dilution) were used. The optical densities of immuno-reactive proteins were detected by Light-Capture AE-6971/2 (ATTO, Tokyo, Japan).

4.5. Accumulation study

The intracellular accumulations of Rho123 in LLC-GA5-COL150 cells for the analysis of P-gp function and calcein in Caco-2 cells for the analysis of MRPs function were measured in the 24-well plates, as reported previously with a slight modification (Murakami et al. 2002b; Patanasethanont et al. 2007a, 2007b). Briefly, Rho123 and calcein-AM were dissolved in pH 7.4 PBS at a concentration of 20 and 2 μM, respectively. After removal of the culture medium, each well was washed twice with 1 ml of PBS, and the cells were preincubated with 300 μl of PBS in the absence or presence of urine extracts or transporter inhibitors at 37 °C for 15 min. Then, Rho123 or calcein-AM with or without urine components or transporter inhibitors was added to each well and the cells were incubated at 37 °C. After the incubation for appropriate period of time, the medium was removed by aspiration and the cells were washed out rapidly twice with 1 ml of ice-cold PBS. The cells were scraped with a rubber policeman and collected in 200 μl of 0.1% (v/v) Triton X-100 in 1 mM HEPES/Tris. The cells were lysed by vortex-mixing and the homogenate was allowed to stand for 1 h. After centrifugation at 9,570 *g* for 5 min, the supernatant was used to measure fluorescence intensity and protein contents. In the inhibition studies, verapamil (300 μM, a P-gp inhibitor), MK-571 (25 μM, a MRPs inhibitor), or urine extracts was added to the uptake medium. In the accumulation study, the concentration of hydrophobic urine components in the uptake medium was adjusted to the same concentration as that in original urine. The concentrations of Rho123 and calcein in cells were determined by microplate fluorometer (Perkin Elmer, Waltham, MA, USA) at wavelengths of 485 nm for excitation and 535 nm for emission. The concentration of protein in each sample was measured by Bradford method using bovine γ-globulin as the standard (Bradford 1976). The accumulation of each fluorescent probe into cells was normalized by dividing the fluorescence intensity by protein contents in each well.

4.6. Statistical analysis

All data are shown as the mean ± standard error of the mean (S.E.M.). Differences among group mean values were assessed by Kruskal-Wallis test followed by post-hoc test (Tukey test). A difference of $P < 0.05$ was considered statistically significant.

Acknowledgement: This work was financially supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (No. 22590162).

This research paper was presented during the 9th Conference on Retrometabolism Based Drug Design and Targeting, May, 12–15, 2013, Orlando, FL, USA.

References

Adlercreutz H, Kerstell J, Svangorg A (1967) Simultaneous estimation of plasma cholesterol, total and individual phospholipids, triglycerides, free fatty acids and cortisol, and urinary estrogens, total 17-ketosteroids, individual 11-deoxy-17-ketosteroids, total 17-ketogenic steroids and pregnanediol during the menstrual cycle and in early pregnancy. Ann Med Exp Biol Fenn 45: 285–292.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

Burke CW, Roulet F (1970) Increased exposure of tissues to cortisol in late pregnancy. Br Med J 1: 657–659.

Challis JR, Patrick JE, Campbell K, Natale R, Richardson B (1980) Diurnal changes in maternal plasma oestrone and oestradiol at 30 to 31, 34 to 35 and 38 to 39 weeks gestational age. Br J Obstet Gynaecol 87: 983–988.

Challis JR, Sprague C, Patrick J (1983) Relation between diurnal changes in peripheral plasma progesterone, cortisol, and estriol in normal women at 30–31, 34–35, and 38–39 weeks of gestation. Gynecol Obstet Invest 16: 33–44.

Charuk JH, Reithmeier RA (1992) Interaction of P-glycoprotein with a hydrophobic component of rat urine. Biochem Biophys Res Commun 186: 796–802.

Charuk JH, Loo TW, Clarke DM, Reithmeier RA (1994) Interaction of rat kidney P-glycoprotein with a urinary component and various drugs including cyclosporine A. Am J Physiol 266: F66–75.

Englund G, Rorsman F, Rönnblom A, Karlbom U, Lazorova L, Gråsjö J, Kindmark A, Artursson P (2006) Regional levels of drug transporters along the human intestinal tract: co-expression of ABC and SLC transporters and comparison with Caco-2 cells. Eur J Pharm Sci 29: 269–277.

Essodaigui M, Broxterman HJ, Garnier-Suillerot A (1998) Kinetic analysis of calcein and calcein-acetoxymethylester efflux mediated by the multidrug resistance protein and P-glycoprotein. Biochemistry 37: 2243–2250.

Evers R, Kool M, Smith AJ, van Deemter L, de Haas M, Borst P (2000) Inhibitory effect of the reversal agents V-104, GF120918 and Pluronic L61 on MDR1 Pgp-, MRP1- and MRP2-mediated transport. Br J Cancer 83: 366–374.

Hebert MF, Easterling TR, Kirby B, Carr DB, Buchanan ML, Rutherford T, Thummel KE, Fishbein DP, Unadkat JD (2008) Effects of pregnancy on CYP3A and P-glycoprotein activities as measured by disposition of midazolam and digoxin: a University of Washington specialized center of research study. Clin Pharmacol Ther 84: 248–253.

Huang ZH, Murakami T, Okochi A, Yumoyo R, Nagai J, Takano M (2001) Expression and function of P-glycoprotein in rats with carbon tetrachloride-induced acute hepatic failure. J Pharm Pharmacol 53: 873–881.

Huang ZH, Murakami T, Okochi A, Yumoto R, Nagai J, Takano M (2000) Expression and function of P-glycoprotein in rats with glycerol-induced acute renal failure. Eur J Pharmacol 406: 453–460.

Hunter J, Jepson MA, Tsuruo T, Simmons NL, Hirst BH (1993) Functional expression of P-glycoprotein in apical membranes of human intestinal Caco-2 cells. Kinetics of vinblastine secretion and interaction with modulators. J Biol Chem 268: 14991–14997.

Ichikawa M, Yoshimura A, Furukawa T, Sumizawa T, Akiyama S (1990) Modulators of the multidrug- transporter, P-glycoprotein, exist in the human plasma. Biochem Biophys Res Commun 166: 74–80.

Ichikawa-Haraguchi M, Sumizawa T, Yoshimura A, Furukawa T, Hiramoto S, Sugita M, Akiyama S (1993) Progesterone and its metabolites: the potent inhibitors of the transporting activity of P-glycoprotein in the adrenal gland. Biochem Biophys Acta 1158: 201–208.

Kool M, de Haas M, Scheffer GL, Scheper RJ, van Eijk MJ, Juijn JA, Baas F, Borst P (1997) Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. Cancer Res 57: 3537–4357.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275.

Mülder HS, Pinedo HM, Timmer AT, Rao BR, Lankelma J (1996) Multidrug resistance-modifying components in human plasma with potential clinical significance. J Exp Ther Oncol 1: 13–22.

Murakami T, Yumoto R, Nagai J, Takano M (2002a) Factors affecting the expression and function of P-glycoprotein in rats: drug treatments and diseased states. Pharmazie 57: 102–107.

Murakami T, Fukuda T, Yumoto R, Nagai J, Kuramoto T, Takano M (2002b) Interaction of endogenous compounds in human and rat urine with P-glycoprotein. Drug Metab Pharmacokinet 17: 245–252.

Murakami T, Takano M (2008) Intestinal efflux transporters and drug absorption. Expert Opin Drug Metab Toxicol 4: 923–939.

Mutsaers HA, van den Heuvel LP, Ringens LH, Dankers AC, Russel FG, Wetzels JF, Hoenderop JG, Masereeuw R (2011) Uremic toxins inhibit transport by breast cancer resistance protein and multidrug resistance protein 4 at clinically relevant concentrations. PLoS One 6: e18438.

Orlowski S, Mir LM, Belehradek J Jr, Garrigos M (1996) Effects of steroids and verapamil on P-glycoprotein ATPase activity: progesterone, desoxycorticosterone, corticosterone and verapamil are mutually non-exclusive modulators. Biochem J 317: 515–522.

Patanasethanont D, Nagai J, Matsuura C, Fukui K, Sutthanut K, Sripanidkulchai BO, Yumoto R, Takano M (2007a). Modulation of function of multidrug resistance associated-proteins by Kaempferia parviflora extracts and their components. Eur J Pharmacol 566: 67–74.

Patanasethanont D, Nagai J, Yumoto R, Murakami T, Sutthanut K, Sripanidkulchai BO, Yenjai C, Takano M (2007b) Effects of Kaempferia parviflora extracts and their flavone constituents on P-glycoprotein function. J Pharm Sci 96: 223–233.

Patrick J, Challis J, Natale R, Richardson B (1979) Circadian rhythms in maternal plasma cortisol, estrone, estradiol, and estriol at 34 to 35 weeks' gestation. Am J Obstet Gynecol 135: 791–798.

Patrick J, Challis J, Campbell K, Carmichael L, Natale R, Richardson B (1980) Circadian rhythms in maternal plasma cortisol and estriol concentrations at 30 to 31, 34 to 35, and 38 to 39 weeks' gestational age. Am J Obstet Gynecol 136: 325–334.

Prime-Chapman HM, Fearn RA, Cooper AE, Moore V, Hirst BH (2004) Differential multidrug resistance-associated protein 1 through 6 isoform expression and function in human intestinal epithelial Caco-2 cells. J Pharmacol Exp Ther 311: 476–484.

Rado A, Crystle CD, Townsley JD (1970) Concentration of estrogens in maternal peripheral plasma in late pregnancy, during labor and post partum. J Clin Endocrinol Metab 30: 497–503.

Rao US, Fine RL, Scarborough GA (1994) Antiestrogens and sterod hormones: substrates of the human P-glycoprotein. Biochem Pharmacol 48: 287–292.

Seithel A, Karlsson J, Hilgendorf C, Björquist A, Ungell AL (2006) Variability in mRNA expression of ABC- and SLC-transporters in human intestinal cells: comparison between human segments and Caco-2 cells. Eur J Pharm Sci 28: 291–299.

Takano M, Hasegawa R, Fukuda T, Yumoto R, Nagai J, Murakami T (1998) Interaction with P-glycoprotein and transport of erythromycin, midazolam and ketoconazole in Caco-2 cells. Eur J Pharmacol 358: 289–294.

Takano M, Yumoto R, Murakami T (2006) Expression and function of efflux drug transporters in the intestine. Pharmacol Ther 109: 137–161.

Tanigawara Y, Okamura N, Hirai M, Yasuhara M, Ueda K, Kioka N, Komano T, Hori R (1992) Transport of digoxin by human P-glycoprotein expressed in a porcine kidney epithelial cell line (LLC-PK1). J Pharmacol Exp Ther 263: 840–845.

Townsley JD, Dubin NH, Grannis G.F, Gartman LJ, Crystle CD (1973) Circadian rhythms of serum and urinary estrogens in pregnancy. J Clin Endocrinol Metab 36: 289–295.

Tulchinsky D, Hobel CJ, Yeager E, Marshall JR (1972) Plasma estrone, estradiol, estriol, progesterone, and 17-hydroxyprogesterone in human pregnancy. I. Normal pregnancy. Am J Obstet Gynecol 112: 1095–1100.

Ueda K, Okamura N, Hirai M, Tanigawara Y, Saeki T, Kioka N, Komano T, Hori R (1992) Human P-glycoprotein transports cortisol, aldosterone, and dexamethasone, but not progesterone. J Biol Chem 267: 24248–24252.

Wils P, Phung-Ba V, Warnery A, Lechardeur D, Raeissi S, Hidalgo IJ, Scherman D (1994) Polarized transport of docetaxel and vinblastine mediated by P-glycoprotein in human intestinal epithelial cell monolayers. Biochem Pharmacol 48: 1528–1530.

Yokooji T, Mori N, Murakami T (2010) Modulated function of tissue efflux transporters under hyperbilirubinemia in rats. Eur J Pharmacol 636: 66–72.

Yokooji T, Mori N, Murakami T (2011) Modulated pharmacokinetics and increased small intestinal toxicity of methotrexate in bilirubin-treated rats. J Pharm Pharmacol 63: 206–213.

Yokooji T, Murakami T, Yumoto R, Nagai J, Takano M (2006) Function of multidrug resistance-associated protein 2 in acute hepatic failure rats. Eur J Pharmacol 546: 152–160.

Yokooji T, Murakami T, Yumoto R, Nagai J, Takano M (2007) Site-specific bidirectional efflux of 2,4-dinitrophenyl-S-glutathione, a substrate of multidrug resistance-associated proteins, in rat intestine and Caco-2 cells. J Pharm Pharmacol 59: 513–520.

Yumoto R, Murakami T, Nakamoto Y, Hasegawa R, Nagai J, Takano M (1999) Transport of rhodamine 123, a P-glycoprotein substrate, across rat intestine and Caco-2 cell monolayers in the presence of cytochrome P-450 3A-related compounds. J Pharmacol Exp Ther 289: 149–155.

Yumoto R, Murakami T, Takano M (2003) Differential effect of acute hepatic failure on in vivo and *in vitro* P-glycoprotein functions in the intestine. Pharm Res 20: 765–771.

Zhao ZY, Xie Y, Fu YR, Li YY, Bogdan A, Touitou Y (2003) Circadian rhythm characteristics of serum cortisol and dehydroepiandrosterone sulfate in healthy Chinese men aged 30 to 60 years. A cross-sectional study. Steroids 68: 133–138.

Zhang H, Wu X, Naraharisetti SB, Chung F, Whittington D, Mirfazaelian A, Unadkat JD (2009) Pregnancy does not increase CYP3A or P-glycoprotein activity in the non-human primate, Macaca nemestrina. J Pharmacol Exp Ther 330: 586–595.