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Novel sample preparation method facilitating identification of urinary drug metabolites by liquid chromatography–tandem mass spectrometry

Hai-Zhi Bu[1], Marzena Poglod, Ronald G. Micetich, Jehangir K. Khan*

Division of Biopharmaceutics and Pharmacokinetics, NAEJA Pharmaceutical, 4290-91A Street, Edmonton, Alberta T6E 5V2, Canada

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Abstract

A simple, efficient procedure was developed for the preparation of urine samples, which greatly facilitated the identification of the urinary metabolites of a new antifungal agent SYN-2836. The urine samples following dilution with acetonitrile (ACN) formed distinct upper (ACN) and lower (aqueous) solution phases. The SYN-2836 metabolites were concentrated in the upper solution except that two glucuronides were concentrated in the lower solution. The upper solutions, containing concentrated metabolites and significantly reduced endogenous polar species, were ideally suitable for the metabolite identification. This novel sample preparation procedure would be applicable in identification of urinary metabolites of other drugs and drug candidates.

Keywords: Sample preparation; Tandem mass spectrometry; Antifungal agents; SYN-2836

1. Introduction

When a drug is administered to animals or humans, the efficacy, action duration and toxicity of the drug are critically dependent on its metabolic pathways in the body [1]. Therefore, the study of drug metabolism plays a key role in pharmaceutical activities such as drug design [1], potency elucidation [2], pharmacokinetic evaluation [1], toxicity assessment [1,3], and clinical trial [4]. Of various qualitative and quantitative studies regarding drug metabolism, structural elucidation of drug metabolites provides vital information that guides selection and optimization of drug candidates.

Identification of drug metabolites in biological matrices is usually a very complicated process, especially for metabolites that exist in trace quantities, are labile and/or have highly diverse polarities. Traditionally, chemical structures of drug metabolites are characterized by following a series of labor-intensive and time-consuming off-line procedures such as extraction, isolation, fractionation and individual structural analysis using spectroscopic techniques [5]. Currently, rapid and simultaneous identification of multiple drug metabolites in biological fluids [6–13] can be readily achieved using highly sensitive, selective and versatile on-line liquid chromatography coupled with atmospheric pressure

*Corresponding author. Fax: +1-780-4610-196.
E-mail address: jkhan@synphar.com (J.K. Khan)
[1]Present address: Department of Metabolic Chemistry, Covance Laboratories, Inc., Madison, WI 53704, USA.

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ionization tandem mass spectrometry (LC–MS–MS) [14].

Sample preparation is an important process in identification of metabolites in various biological matrices such as urine, feces and bile. An ideal sample preparation procedure may require that the number of drug metabolites extracted by the procedure is as many as possible, the concentration of the extracted drug metabolites is as high as possible, and the amount of non-drug-related species remaining in the treated sample is as little as possible. Of various biological matrices, urine is most frequently used for metabolite identification. Urine samples are usually treated with different procedures, for instance, centrifugation, filtration, protein precipitation with acetonitrile (ACN), liquid–liquid extraction, and solid-phase extraction [15]. The methods chosen for sample preparation depend, to a large extent, on the nature of metabolites as well as required sensitivity.

Twelve urinary metabolites of SYN-2836 (Fig. 1), a new antifungal agent, have been identified in dogs using LC–MS and LC–MS–MS approaches [16,17]. In these studies, urine samples were treated with a simple, efficient procedure. Briefly, the urine samples were diluted with ACN or ACN containing 0.5% formic acid (HFo). After mixing and standing, the diluted samples formed upper and lower solution phases. The resultant upper and lower solutions were respectively injected for analysis. In the present study, additional five SYN-2836 metabolites were identified utilizing the same analytical methodologies as used previously [16]. Using all known SYN-2836 metabolites as model compounds, the advantages of the novel sample preparation protocol, over conventional methods, were then evaluated. The partition of

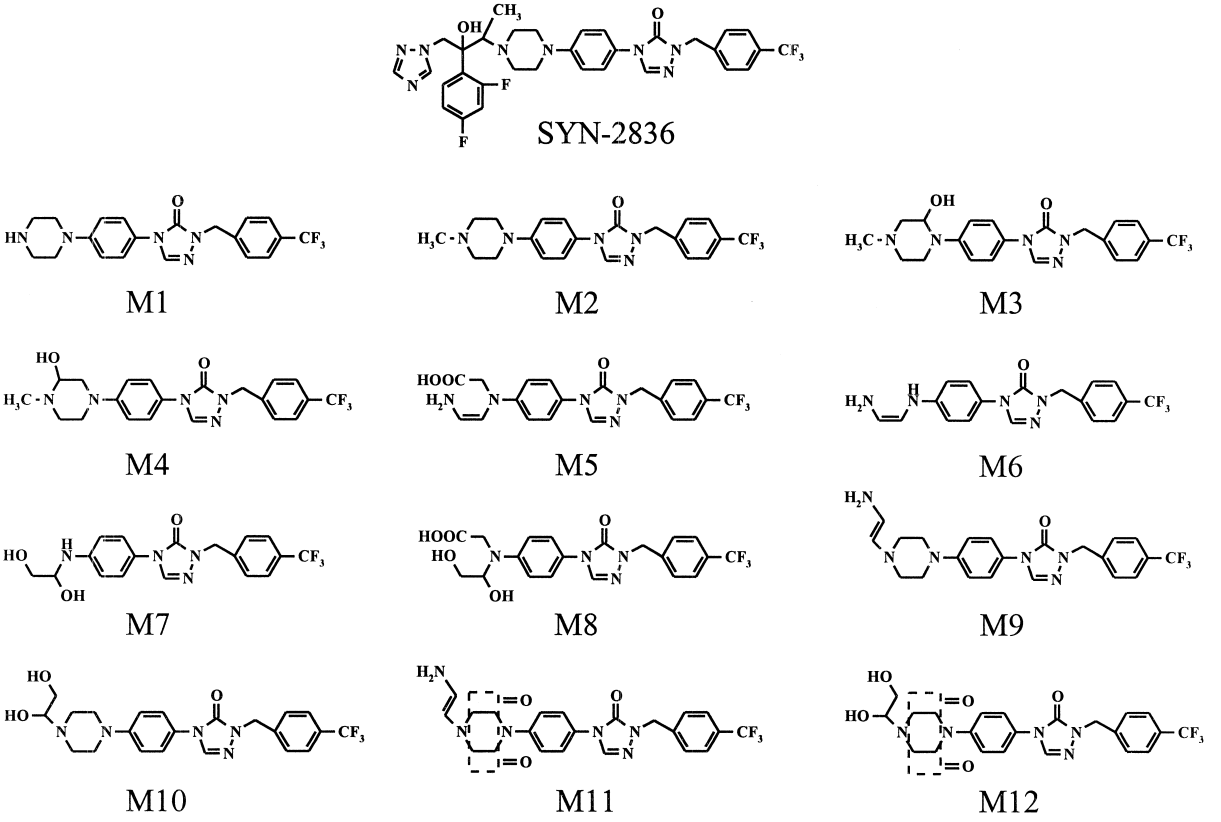

Fig. 1. Chemical structures of SYN-2836 and its metabolites identified in our previous studies [16,17].

the metabolites in the upper and lower phases was largely dependent on their polarities. It was found that endogenous species and two conjugated metabolites (glucuronides) of SYN-2836 mainly remained in the lower solution while all other SYN-2836 metabolites were concentrated in the upper solution. Thus, the upper solutions, containing concentrated metabolites and largely diminished endogenous components, were ideal samples for the identification of the SYN-2836 metabolites. This new, simple and efficient sample preparation procedure would be readily employed for rapid identification of urinary metabolites of other drugs and drug candidates.

2. Experimental

2.1. Materials

SYN-2836 was synthesized at SynPhar Labs. (Edmonton, Canada). HPLC-grade acetonitrile was purchased from EM Science (Gibbstown, NJ, USA), and formic acid was received from BDH (Toronto, Canada).

2.2. Dosing and urine collection

SYN-2836 was dissolved in an aqueous formulation containing hydroxypropyl-β-cyclodextrin (57%, w/v) and hydrochloric acid (0.25 *M*) to a final concentration of 125 mg/ml. Three male beagle dogs were administered multiple oral doses of 30 mg/kg/day in the morning for 14 days. Following the last administration, urine collected over 0–24 h was pooled and used for metabolite identification. Urine collected from the dogs administered with the formulation excluding the drug was used as control.

2.3. Sample preparation

Urine samples or controls were diluted 1:1 (v/v) with ACN or ACN containing 0.5% HFo. The diluted samples were vortex-mixed for 5 min and kept standing for 1 h at ambient temperature (22±2°C), or 4 or −20°C if necessary, to allow formation of upper and lower liquid phases. The upper and lower solutions were respectively transferred to polypropylene microcentrifuge tubes, centrifuged at 3500 *g* for 5 min, and transferred to autosampler vials at 4°C for injection (30 μl).

2.4. LC–MS and LC–MS–MS

Metabolites were separated on a Phenomenex Prodigy C_{18} column (150×2.0 mm, 5 μm, Torrance, CA, USA) with a Waters 2690 separations module system including a built-in autosampler (Milford, MA, USA). Solvent A was ACN containing 0.1% HFo and solvent B was water containing 0.1% HFo. The components were eluted using the following linear gradient: 0 min, 100% B; 20 min, 80% A; 30 min, 80% A; 31 min, 100% B; 38 min, 100% B; flow 0.2 ml/min. An electronic valve actuator (Jones Chromatography, Wales, UK) with a Rheodyne 7010 selector valve (Cotati, CA, USA) was used to divert the flow to waste, when no data acquisition was taking place, in order to minimize contamination of the Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, UK). The effluent from the actuator was split to allow 50 μl/min entering the electrospray interface (source temperature 100°C, capillary voltage +3.5 kV, cone voltage 35 V). Nitrogen served as the drying and nebulizing gas at flow-rates of 250 and 35 l/h, respectively. For collision induced dissociation (CID) fragmentation, argon was used as the target gas at a pressure of $5 \cdot 10^{-4}$ mbar and collision energy was set at 20 or 30 eV depending on compounds of interest. Full scan LC–MS was performed from 8 to 30 min over the *m/z* range 200–700. Product ion scan LC–MS–MS was carried out from (t_R−2) to (t_R+2) min (t_R: retention time of a selected ion) over the *m/z* range from 50 to an adequate value for the selected ion. All instrumental controls and data processing were performed with Micromass Masslynx software 3.2.

3. Results and discussion

3.1. Metabolite identification

Exclusively using LC–MS and LC–MS–MS as analytical tools, metabolite identification can be regarded as a process of structural elucidation of potential drug metabolites by interpretation of product ion mass spectra of selected precursor ions,

guided by concept of well-known biotransformation pathways. At present, LC–MS and LC–MS–MS methods are most widely used for rapid identification of drug metabolites in trace amount, however, it is highly challenging to achieve unequivocal structural characterization without the use of other analytical techniques (e.g., nuclear magnetic resonance). In practice, confidence of structural elucidation achieved using LC–MS and LC–MS–MS remarkably relies on how to strategically use the tools.

In this study, the five additional SYN-2836 metabolites, M13, M14 and M15 (Fig. 2) along with M16 and M17 (Fig. 3), were identified by executing the same strategies as used previously [16,17]. M13 was possibly formed via *N*-dealkylation of SYN-2836 or M1 (Fig. 1), and its chemical structure was supported by interpretation of its product ion mass spectrum (Fig. 2A). Similarly, the structure of M14 was proposed in terms of the major similarity and minor difference between the product ion mass spectra of M13 and M14 (Fig. 2A vs. Fig. 2B), which was also supported by a convincing interpretation of its product ion mass spectrum (Fig. 2B). M14 could be formed via hydroxylation and alcohol-to-ketone oxidation of M13. The structure of M15 was possibly formed via oxidative cleavage of SYN-2836 followed by ketone-to-alcohol reduction, which was confirmed by interpretation of its product ion mass spectrum (Fig. 2C) along with the use of its authentic compound (data not shown).

In the product ion mass spectrum of M16 (Fig. 3A) the product ion at m/z 226 represents a neutral loss of 176 Da (i.e., $C_6H_8O_6$) from the protonated precursor ion at m/z 402, suggesting that M16 is a glucuronide conjugate of M15 (Fig. 3A vs. Fig. 2C). Likewise, the product ion at m/z 379 in the product ion mass spectrum of M17 (Fig. 3B) was generated by the neutral loss of 176 Da from the protonated molecule at m/z 555, indicating M17 a glucuronide of an intermediate alcohol with a molecular mass of 378. The structure of the intermediate was easily postulated by interpretation of the product ion mass spectrum of M17 (Fig. 3B). In the prediction of possible SYN-2836 metabolites, the intermediate alcohol was expected to be a metabolite. However, this species preferred to conjugate with glucuronic acid, resulting in itself not being present (or not detected) as a stable metabolite.

3.2. Novel sample preparation

ACN is one of the most commonly used solvents in the preparation of urine samples for LC–MS and LC–MS–MS analysis. Nonetheless, no studies have ever been reported, to the best of our knowledge, regarding the formation of upper and lower solution phases when urine samples are extracted with ACN. In this study, the distinct upper and lower phases were fortuitously observed when the dog urine samples were diluted 1:1 (v/v) with ACN. The volume ratios of upper to lower phases ($R=V_{up}/V_{low}$, V_{up} and V_{low} represent the volumes of upper and lower solutions, respectively) were dependent on the temperatures at which the extraction was performed. The R values ranged from 0.27 to 0.40 at room temperature (22±2°C) and from 0.45 to 0.51 at 4°C, indicating higher R values at lower temperature. Nevertheless, no significant further increase in R values was observed at −20°C. In addition, the R values were also dependent on the acidity of the extraction solvent. No significant changes in R values were observed using ACN containing 0.5% HFo, however, much smaller R values (or even no phase isolation in some cases) observed using ACN containing 2% HFo, compared to using ACN only.

Interestingly, no phase isolation was observed when methanol, ethanol, isopropanol and acetone were used as the extraction solvents at room temperature, or even at lower temperature (e.g., 4 or −20°C). Of the five highly water-miscible organic solvents used in the study, only ACN molecule has no ability to form hydrogen bonding with molecules of water and various biological species in urine. This suggests that the incapability of ACN molecule forming hydrogen bonding may be the major reason for the formation of upper and lower solutions of urine extracted by ACN. Other factors related to the phase isolation were not explored in this work.

Most of endogenous components in urine are highly water-soluble chemical and biochemical species (e.g., organic acids, bases and salts, carbohydrates and proteins) or exclusively water-soluble compounds (e.g., inorganic salts or electrolytes). As expected, these highly water-soluble species were preferably distributed in the lower (aqueous) solution of the extracted urine sample, resulting in the lower solution very dirty considering its dark or brownish

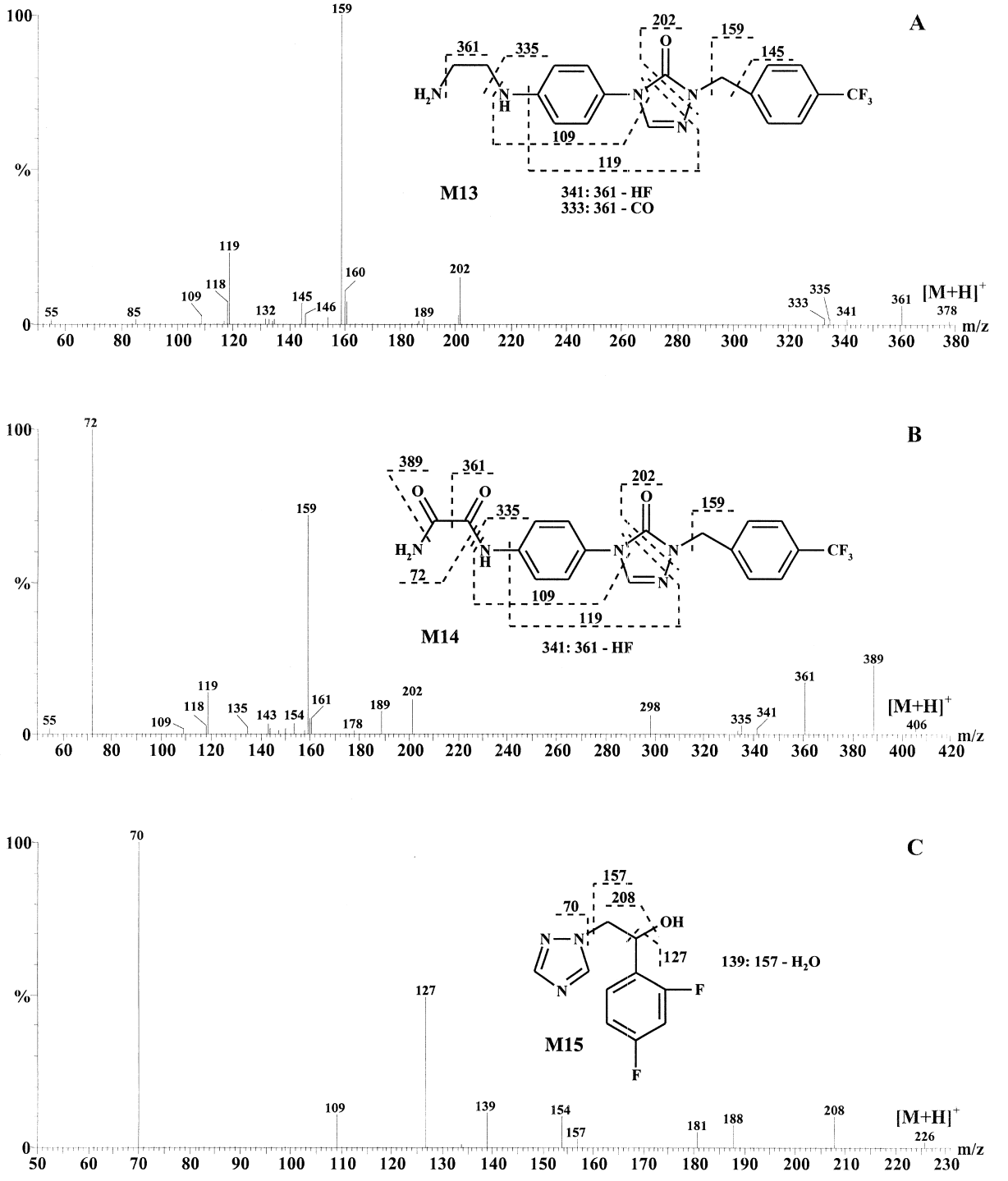

Fig. 2. LC–MS–MS product ion mass spectra of protonated SYN-2836 metabolites M13 (A) at m/z 378, M14 (B) at m/z 406 and M15 (C) at m/z 226, acquired by injection of the upper solution of the extracted urine with acetonitrile.

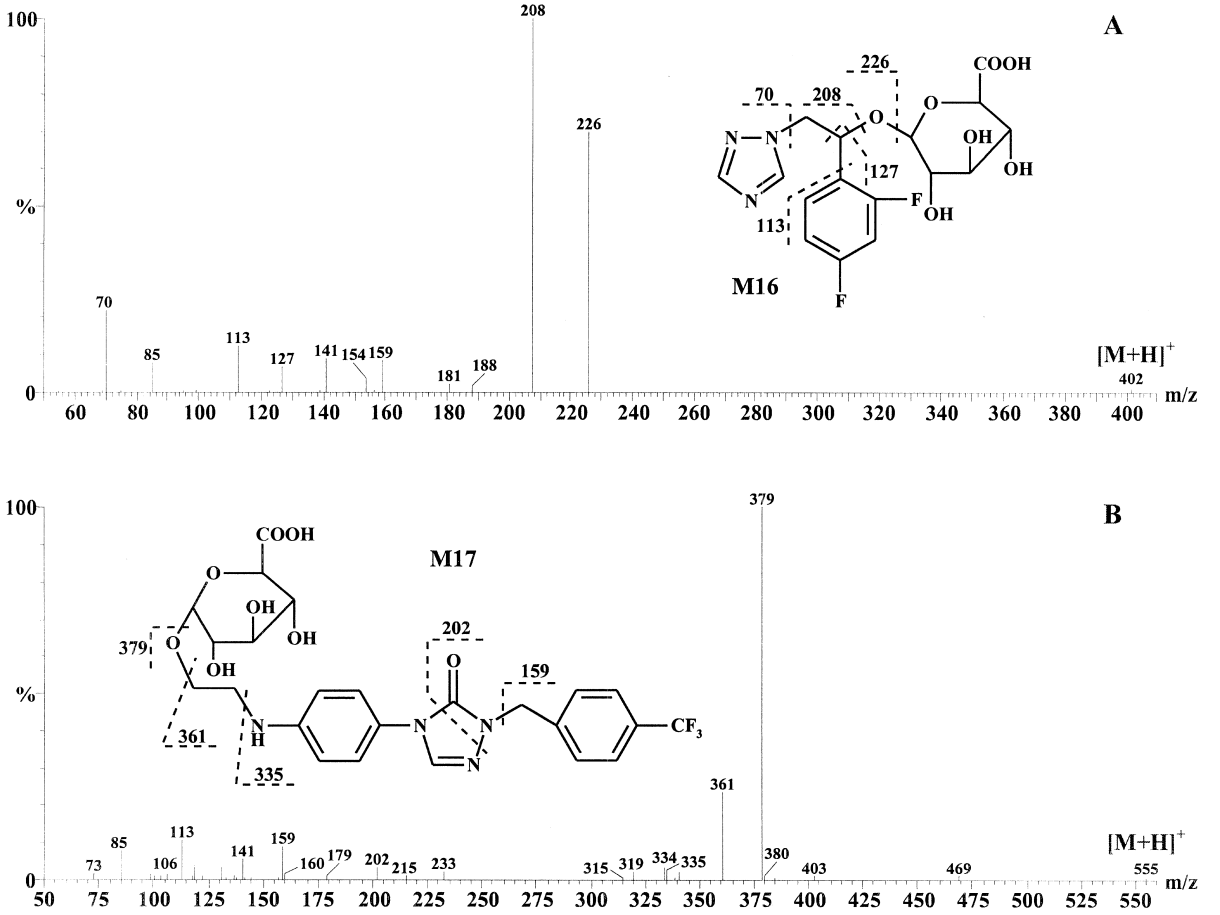

Fig. 3. LC–MS–MS product ion mass spectra of protonated SYN-2836 metabolites M16 (A) at m/z 402 and M17 (B) at m/z 555, acquired by injection of the lower solution of the extracted urine with acetonitrile.

yellow color accompanying the presence of precipitates and suspended particles. In contrast, the upper (ACN) solution was very clean in view of its pale yellow color along with the absence of precipitates and suspended particles. In addition, the upper solution possibly contained no or minimal inorganic salts. Therefore, the upper solution was ideally suited for LC–MS and LC–MS–MS analysis.

As shown in Table 1, the reconstructed ion chromatographic peak area ratios (r) of the SYN-2836 metabolites in the upper phase to them in the lower phase range from 0.22 to 10.21, when the urine samples were treated by ACN, and from 0.21 to 8.70 when the urine samples were extracted by ACN containing 0.5% HFo. The metabolites M1–M15 were concentrated in the upper solution considering their r values >1, while M16 and M17 (two glucuronides) concentrated in the lower solution in view of their r values <1 (Table 1), which was consistent with their polarities. As an extreme case, the parent SYN-2836, a highly lipophilic compound, was detectable in the upper solution but not in the lower solution. It is also seen that addition of HFo (0.5%) to ACN affected the peak ratio values but only significantly for M5 and M8, two carboxylic acids (Table 1). In addition, it was proved that most of the endogenous species detected by LC–MS were greatly concentrated in the lower solution (data not shown). Since ACN is an ideal solvent for LC–MS and LC–MS–MS analysis, removal of the solvent from the treated samples followed by reconstitution of the resulting residues, as usually performed in a

Table 1
Partition of SYN-2836 and its metabolites in upper and lower solution phases following extraction of urine samples with acetonitrile (ACN) or ACN containing 0.5% formic acid (HFo)

Compound	Peak area ratio Upper phase to lower phase		Compound	Peak area ratio Upper phase to lower phase	
	ACN[a]	ACN (0.5% HFo)[b]		ACN[a]	ACN (0.5% HFo)[b]
SYN-2836	2764/ND[c]	2589/ND	M9	4.86	4.43
M1	2.08	2.31	M10	2.48	3.06
M2	3.02	3.00	M11	8.28	8.03
M3	2.43	3.82	M12	6.36	5.92
M4	4.90	4.97	M13	2.40	2.03
M5	1.58	6.91	M14	7.62	8.70
M6	10.21	8.58	M15	1.67	2.42
M7	3.61	3.74	M16	0.22	0.21
M8	1.24	5.28	M17	0.86	0.95

[a] Urine samples extracted with ACN.
[b] Urine samples extracted with ACN containing 0.5% HFo.
[c] SYN-2836 not detectable in lower phase.

conventional extraction process, was eliminated in the present procedure. These results indicate that the simple, efficient urine sample preparation method developed in this study well meets the requirements of an ideal sample preparation as stated above. It is anticipated that this novel method can be used for identification of metabolites of other drugs in urine. Note that this method is suitable for qualification but may not be suitable for quantification due to the inconstant *R* values from sample to sample.

4. Conclusions

Novel liquid–liquid extraction of urine with ACN or ACN containing 0.5% HFo has been achieved. Such a simple, efficient sample preparation procedure has greatly facilitated the identification of urinary primary and secondary metabolites of SYN-2836. Considering the significant variation in the volume ratios of upper to lower solution phases between urine samples, this procedure may lack applicability for quantification of urinary metabolites.

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