Research Paper

Crystalline Form Information from Multiwell Plate Salt Screening by Use of Raman Microscopy

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Purpose. The purpose of this study was to establish a useful methodology, possibly providing information on the stoichiometry of pharmaceutical drug salts obtained from salt screening by using a multiwell plate and a Raman microscope.

Methods. Tamoxifen salt screening was conducted with monobasic and polybasic acids on 96-well quartz plates with a Raman microscope. Appearance and crystalline forms of salts prepared on 96-well plates were observed by polarizing light microscope and Raman microscope, respectively. Based on the results of the salt screening, tamoxifen citrate and fumarate salts were prepared on a large scale. The salts prepared were characterized by powder X-ray diffractometry (PXRD) and ion chromatography.

Results. The results of the multiwell salt screening indicated that tamoxifen has a tendency toward the formation of mono salt as opposed to hemi salt with polybasic acid, and that most of tamoxifen salts gave several potential polymorphic forms. PXRD patterns of scaled-up tamoxifen citrate and fumarate salts suggested that the same crystalline form was obtained from the binary mixture regardless of molar ratios of 2:1 or 1:1 (tamoxifen/acid). The crystalline forms obtained were tamoxifen monocitrate and monofumarate salts as measured by ion chromatography.

Conclusions. Salt screening on multiwell plates with a Raman microscope provided novel insight into the characteristics prediction of the stoichiometrical salts in addition to potential polymorph information. Based on the stoichiometrical information of salts, the amount of compound and time required for crystalline form selection of drug candidates would be significantly reduced.

KEY WORDS: crystalline form; polymorphism; Raman microscope; salt screening; tamoxifen.

INTRODUCTION

In the field of drug development, solid form selection of pharmaceutical compounds, such as crystalline and amorphous, is an essential process, because selected solids can dominate physicochemical properties affecting the efficacy, safety (1–3), stability (4), manufacturing process (5,6), and quality control (7). Especially, crystalline form selection has gotten a lot of attention recently. For example, inadequate crystalline form selections have resulted in the withdrawal of products from the market (8,9). Moreover, insufficient crystalline form screening has led to patent litigation (10). To avoid these problems, many articles have discussed a crystalline form selection strategy comprised of salt screen-ing, polymorph screening, and crystalline form characterization (4,11–18).

Although significant progress in the computational prediction of polymorphs has been made in the past decade (19), the packing structure of crystals in salt and pseudopolymorphic systems, as yet, cannot be predicted, and experimental salt and polymorph screening is still relied on. To reveal or validate polymorphs, numerous studies dealing with high-throughput salt and polymorph screening have been conducted because polymorph or pseudopolymorph would be given under the wide range of solvent and crystallization conditions (20–29). High-throughput salt and polymorph screening is commonly performed using a multiwell plate, powder X-ray diffractometry (PXRD), and a Raman microscope. Raman microscopy is especially useful for salt screening because the technique has made it possible to obtain not only physical information but also chemical information (30,31). The flood of data provided by high-throughput equipment can be automatically analyzed by computer using classification software (32–34). These techniques for crystalline form identification from physical information have been currently applied to high-throughput salt and polymorph screening. However, chemical information specific to high-throughput salt and polymorph screening

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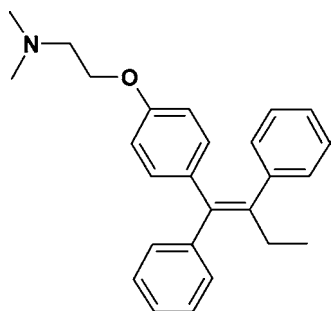[3] To whom correspondence should be addressed. (e-mail: takashi.kojima@pfizer.com)

Fig. 1. Chemical structure of tamoxifen.

with a Raman microscope has not been fully elucidated up to now.

This paper focuses on salt screening through the use of multiwell plates and Raman microscopy using tamoxifen (Fig. 1) as a model drug. Tamoxifen is clinically used as an antiestrogenic agent for the treatment of breast cancer (35) and marketed as a monocitrate salt.

In this study, we performed effective salt screening on tamoxifen by using Raman microscopy to evaluate chemical information. Some of the salts obtained were also separately prepared on a large scale, characterized, and compared with the results of salt screening on multiwell plates using a Raman microscope. In addition, information obtained by using this combination technique was discussed.

MATERIALS AND METHODS

Materials

Tamoxifen free base was obtained from Aldrich Chemical Company (Milwaukee, WI, USA). Methanesulfonic acid was obtained from Nacalai Tesque (Kyoto, Japan), and other organic acids were obtained from Wako Pure Chemical Industries (Osaka, Japan). All solvents were purchased from Wako Pure Chemical Industries.

Salt Screening on 96-Well Plate

Tamoxifen salt formation on a 96-well plate was conducted using a combination of 12 kinds of crystallization solvents and six different acids according to the method reported (25). Each methanol solution of tamoxifen and acids, which were methanesulfonic acid and benzenesulfonic acid as monobasic acids, and L-tartaric acid, fumaric acid, citric acid, and succinic acid as polybasic acids, was prepared in the same concentration of 20 mM prior to use. Tamoxifen solution (50 μl) was placed in all wells of the 96-well quartz plate (Hellma, Müllheim, Germany). Each aqueous solution of monobasic acid (50 μl) was added into each row of the plate to give the binary mixture of tamoxifen and acid in a molar ratio of 1:1. Each aqueous solution of polybasic acid (25 or 50 μl) was added to give 2:1 or 1:1 binary mixtures of tamoxifen and acid in the same manner as monobasic acid solution. The plate was sealed with CAPMATS (Whatman, Brentford, UK) and was shaken with BioShaker M·BR-022 (TAITEC, Saitama, Japan) at room temperature for 4 h.

Solvent was evaporated under reduced pressure at 40°C overnight. Each crystallization solvent, 200 μl of methanol, ethanol, isopropyl alcohol, acetonitrile, acetone, ethyl acetate, isopropyl ether, tetrahydrofuran, toluene, dichloromethane, cyclohexane, and 100 μl of water, was added to each column of the plate. The plate was sealed again and shaken at 40°C for 4 h and allowed to stand at room temperature overnight. All solvents in the plate were evaporated slowly in an atmosphere of nitrogen to attempt crystallization. Solids recrystallized on the 96-well plate were checked for crystallinity with a polarizing light microscope (PLM) and analyzed with Raman microscope ($n = 2$ in each well).

Salt Preparation

Tamoxifen fumarate and citrate salts were prepared on a 300-mg scale. Tamoxifen free base was dissolved in acetonitrile, and fumaric or citric acid was added to give 1:1 and 2:1 (tamoxifen/acid) mixtures. The suspension obtained was dried under reduced pressure. The resultant products were recrystallized by slowly cooling the saturated solution from the same 12 solvents as those for the salt screening on the 96-well plate, filtrated and dried in an atmosphere of nitrogen. Crystals obtained were subjected to PXRD and ion chromatographic analysis.

Raman Microscopy

Raman spectra were recorded on a LabRam HR-800/HTS-Multiwell (Jobin Yvon Horiba, Edison, NJ, USA) at room temperature, equipped with a backscattering light path system of a light-emitting diode laser (785 nm, 300 mW) as an excitation source and an air-cooled charge-coupled device detector. A 20-fold superlong working distance objective lens was used to collect the backscattered light. The spectra were acquired with 5.84 cm^{-1} spectral width and at least 30 s exposure. The laser power incident on the sample was 87mW. The spectrometer was calibrated with a silicon wafer.

Powder X-Ray Diffractometry

Powder X-ray diffraction patterns were collected using an RINT-TTR (Rigaku, Tokyo, Japan) with Cu Kα radiation generated at 300 mA and 50 kV. Samples were placed on an aluminum rotation plate and rotated at 60 rpm at room temperature. Data were collected from 3 to 35 (2θ) at a step size of 0.02° and scanning speed of 4°/min.

Ion Chromatography

Ion chromatography was performed using a Dionex (Sunnyvale, CA, USA) DX 500 apparatus equipped with a GP50 pump and an AS50 autosampler, connected to a CD25 conductivity meter with Peaknet software. Potassium hydroxide eluent was generated electrolytically using a Dionex EG40 eluent generator, and multistep gradient concentrations ranging from 10 to 60 mM allowed the complete separation of counterions in aqueous media in 15ômin at a constant flow rate of 1.0 ml/min. An ASRS Ultra II suppressor at 100 mA, with an external water feed, was used for anion chromatography.

Table I. Raman Spectra Classification of Tamoxifen Salts on 96-Well Plates

Counter acids	TAM : CA	Solvents											
		MeOH	EtOH	IPA	MeCN	Acetone	EtOAc	IPE	THF	Toluene	DCM	cHex	Water
1 Methanesulfonic acid[a]	1 : 1	M1	M1	M1	M1	M1	M1	M1	M1	M2	M2	M1	–
2 Benzenesulfonic acid[b]	1 : 1	–	B2	B1	B2	–	B1	B1	–	B2	B2	B1	B2
3 L-Tartaric acid[c]	1 : 1	T1	T1	T1	T1	T1	T1	T3	T1	T1	T2	T1	T1
4 L-Tartaric acid[c]	2 : 1	T2	T1	T2 Free	T1	T2	T2	T2 Free	T1	T1	T1	T2	T2
5 Fumaric acid[d]	1 : 1	F2	F1	F1	F1	F1	F1 F2	F2	F2	F2 CA	F2	F2	F1
6 Fumaric acid[d]	2 : 1	F1	F1	F1	Free	F1	–	F2	–	F2	–	–	F1
7 Citric acid[e]	1 : 1	C1	C1	C1	C1	C1	C1	C1	C1	C2	–	C1	C1
8 Citric acid[e]	2 : 1	C1	C1	C1	C1	–	Free	C2	–	C2	–	C1	C1
9 Succinic acid[f]	1 : 1	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1
10 Succinic acid[f]	2 : 1	S1	S1	S1	S1	S1 Free	S1	S1	S1	S1	–	S1	S1
TAM (free base)[g]	–	Free	Free	Free	Free	–	–	Free	Free	Free	–	–	–

Abbreviations used in the table; MeOH, methanol; EtOH, ethanol; IPA, isopropyl alcohol; MeCN, acetonitrile; EtOA, ethyl acetate; IPE, isopropyl ether; THF, tetrahydrofuran; DCM, dichloromethane; cHex, cyclohexane; TAM, tamoxifen; CA, counter acid.

[a] Raman spectra of crystals formed with TAM and methanesulfonic acid (1:1) were classified as M1 or M2.
[b] Raman spectra of crystals formed with TAM and benzenesulfonic acid (1:1) were classified as B1 or B2.
[c] Raman spectra of crystals formed with TAM and L-tartaric acid (1:1, 2:1) were same patterns and classified as T1, T2 or T3.
[d] Raman spectra of crystals formed with TAM and fumaric acid (1:1, 2:1) were same patterns and classified as F1 or F2.
[e] Raman spectra of crystals formed with TAM and citric acid (1:1, 2:1) were same patterns and classified as C1 or C2.
[f] Raman spectrum of crystals formed with TAM and succinic acid (1:1, 2:1) was just one pattern and classified as S1.
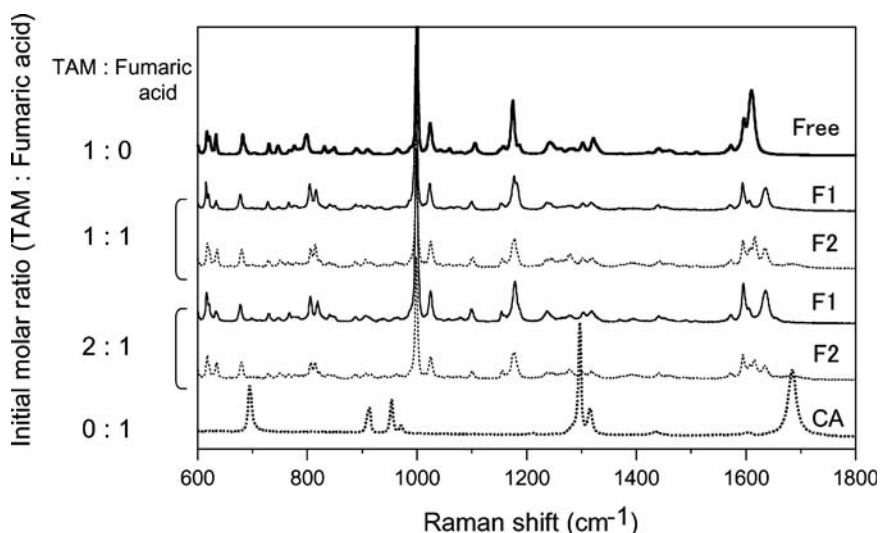[g] Raman spectrum of TAM crystals was just one pattern and classified as Free.

Fig. 2. Raman spectra of tamoxifen (TAM), TAM fumarate, and fumaric acid crystals. TAM fumarate was prepared with TAM and fumaric acid (1:1 and 2:1) on a 96-well plate. Free, F1, F2, and counter acids (CA) correspond to the classification in Table I. Heavy solid line (—), TAM (free); solid line (—), TAM fumarate (F1); dotted line (┈), TAM fumarate (F2); and heavy dotted line (▬), fumaric acid (CA).

RESULTS

Chemical Information from Salt Screening on 96-Well Plate

Polarizing light microscope observations indicated that 114 crystalline solids were obtained out of 132 wells on the 96-well plates. Except for amorphous or oily substances observed by PLM, crystals obtained were analyzed by Raman microscope. The results of Raman microscopy for tamoxifen salts on the 96-well plate are shown in Table I. The Raman spectra of all crystals were sorted in comparison with each pattern in the shift region 600–1800 cm^{-1}. Sorted spectra were compared with those of the tamoxifen free base and counter acids measured separately. Crystals from the free base and counter acids detected by Raman microscopy are hereafter denoted as free and CA, respectively. Crystalline forms in the binary mixtures of tamoxifen and acid on the plate identified as salts showing different spectra are presented numerically with initial, i.e., M1 and M2 for two crystalline forms of tamoxifen mesylate salt.

Raman spectra indicated that salt screening revealed that only one crystalline form was obtained for both tamoxifen free base and succinate salt, whereas two potential polymorphic forms were obtained for tamoxifen mesylate, besylate, fumarate, and citrate salts, and three potential polymorphic forms were obtained for tamoxifen L-tartrate salt, including hydrate or solvate. In addition to potential polymorph information, the same crystalline forms were detected on the wells in molar ratios of 1:1 and 2:1 (tamoxifen/polybasic acid).

Chemical identification of solids was also performed by Raman microscope. Tamoxifen free base was detected on the wells in a molar ratio of 2:1 (tamoxifen/L-tartaric acid) in
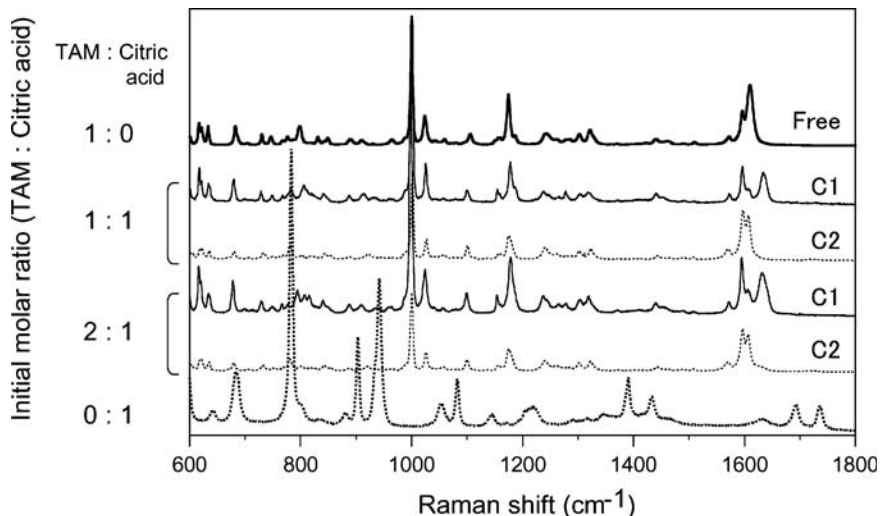

Fig. 3. Raman spectra of TAM, TAM citrate, and citric acid crystals. TAM citrate was prepared with TAM and citric acid (1:1 and 2:1) on a 96-well plate. Free, C1, and C2 correspond to the classification in Table I. Heavy solid line (—), TAM (free); solid line (—), TAM citrate (C1); dotted line (┈), TAM citrate (C2); and heavy dotted line (▬), citric acid.
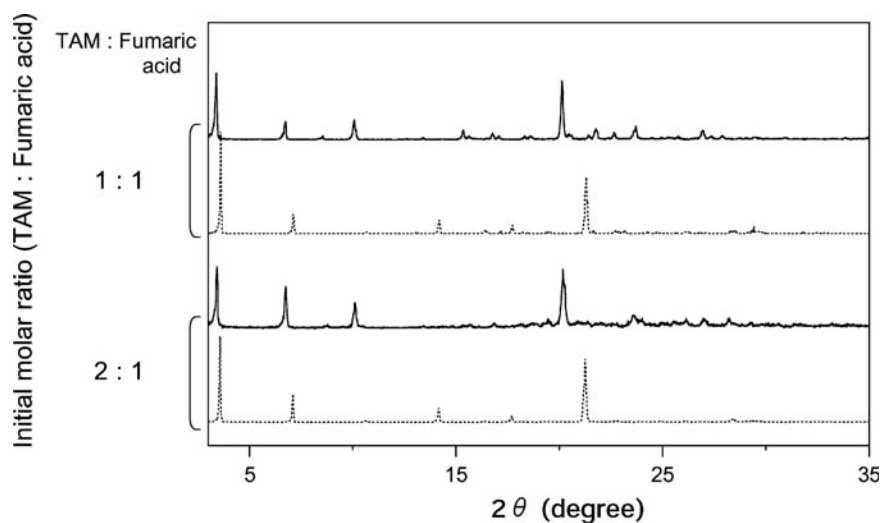
Fig. 4. Powder X-ray diffractometry (PXRD) patterns of TAM fumarate prepared with TAM and fumaric acid (1:1 and 2:1) on a large scale. Solid line (—), TAM fumarate form A; dotted line (·····), TAM fumarate form B.

isopropyl alcohol, 2:1 (tamoxifen/L-tartaric acid) in isopropyl ether, 2:1 (tamoxifen/fumaric acid) in acetonitrile, 2:1 (tamoxifen/citric acid) in ethyl acetate, and 2:1 (tamoxifen/succinic acid) in acetone. Fumaric acid as a counter acid was detected in the well in a molar ratio of 1:1 (tamoxifen/fumaric acid) in toluene.

The Raman spectra of crystals in the wells with combinations of tamoxifen and fumaric acid are shown in Fig. 2. The Raman spectra of F1 in molar ratios of 1:1 and 2:1, as presented in Table I, showed the same patterns with distinct peaks at 1595 and 1638 cm^{-1}. The Raman spectra of F2 in molar ratios of 1:1 and 2:1 showed the same patterns with distinct peaks at 1596, 1618, and 1637 cm^{-1}. These results suggested that these crystals were identified as tamoxifen fumarate because their spectra were different from the spectra of either free base with a distinct peak at 1613 cm^{-1} or fumaric acid with the distinct peak at 1686 cm^{-1}.

The Raman spectra of the crystals in the wells with combinations of tamoxifen and citric acid are shown in Fig. 3. Raman spectra of C1 in molar ratios of 1:1 and 2:1, as

presented in Table I, showed the same patterns with distinct peaks at 1595 and 1635 cm^{-1}. Raman spectra of C2 in molar ratios of 1:1 and 2:1 showed the same patterns with the distinct peaks at 1598 and 1608 cm^{-1}. These results suggested that these crystals were identified as tamoxifen citrate because their spectra were different from the spectra of either free base with a distinct peak at 1613 cm^{-1} or citric acid with distinct peaks at 1693 and 1735 cm^{-1}.

These results indicated that tamoxifen would prefer to form salts in a 1:1 molar ratio (tamoxifen/polybasic acid), and the excess amount of nonsalt-forming tamoxifen free base tended to be detected in the rows of the plate displaying a binary mixture of tamoxifen and polybasic acid in a molar ratio of 2:1.

Characterization of Salts by Scaled-Up Preparation

Tamoxifen fumarate and citrate prepared on a 300-mg scale were characterized using PXRD and ion chromatogra-
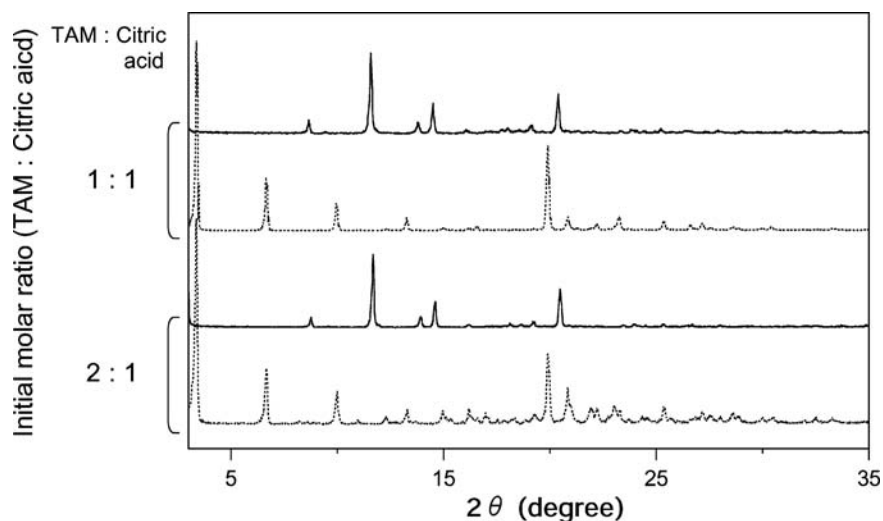

Fig. 5. PXRD patterns of TAM citrate prepared with TAM and citric acid (1:1 and 2:1) on a large scale. Solid line (—), TAM citrate form A; dotted line (·····), TAM citrate form C.

phy. Tamoxifen fumarate prepared in molar ratios of 1:1 or 2:1 (tamoxifen/fumaric acid) was recrystallized in 12 solvents, and two crystalline forms were detected by PXRD in each preparation. PXRD patterns of these crystalline forms assigned as forms A and B, which were prepared in a 1:1 molar ratio, were confirmed to be the same as forms A and B prepared in a 2:1 molar ratio, respectively (Fig. 4). Ion chromatography indicated that the molar ratios of fumaric acid to tamoxifen free base were 1.02 and 1.03 for tamoxifen fumarate prepared in molar ratios of 1:1 and 2:1 (tamoxifen/fumaric acid), respectively. These results clearly suggested that crystals obtained were tamoxifen monofumarate.

Tamoxifen citrate was prepared in the same manner as tamoxifen fumarate, and at least two crystalline forms were detected by PXRD. Because forms A and B were the forms already reported (36), the new form was designated as form C. The PXRD patterns of the two crystalline forms assigned as forms B and C, which were prepared in a 1:1 molar ratio, were found to be the same as forms B and C prepared in a 2:1 molar ratio, respectively (Fig. 5). Ion chromatography indicated that the molar ratios of citric acid to tamoxifen were 1.01 and 0.99 for tamoxifen citrate prepared in molar ratios of 1:1 and 2:1 (tamoxifen/citric acid), respectively. These results suggested that the crystals obtained were tamoxifen monocitrate.

Characterization of the salts produced in the scaled-up preparation gave the same stoichiometrical information on tamoxifen fumarate and citrate salts as salt screening using the 96-well plates. These results suggested that salt screening with the 96-well plates would provide not only potential polymorph information but also prediction of stoichiometrical information on the salts.

DISCUSSION

We have first demonstrated that salt screening using multiwell plates and a Raman microscope can suggest stoichiometrical information on polyprotic salts. Salt screening using multiwell plates could be satisfactorily performed with less than 100 mg of drug candidate, and stoichiometrical information was easily obtained by comparing the Raman spectra of a drug candidate in the free base and counter acid condition, the free acid and counter base condition, and the salts found on the multiwell plates.

In the process of drug development, Raman microscopes have been widely used as an analytical tool for the chemical and physical identifications of either or both drugs and contaminants within pharmaceutical systems, which is also referred to as mapping of the dosage form (37,38). Physical indication, focusing on polymorph detected by Raman microscopy in place of PXRD, has been increasingly reported over the last few years (39). Attention has been also drawn to polymorphic evaluation by Raman microscopy of salt and polymorph screening of pharmaceutical drug molecules (22,24). However, to the best of our knowledge, chemical information obtained by Raman microscopy was not discussed.

In salt screening with Raman microscopy, it is possible to detect drug molecules, counter acids/bases, and salts individually. In salt formation of ionic drug molecules with polyprotic counterions, it is possible to form salt in more than one stoichiometric combination. Some ionic drug candidates form only one stoichiometric salt in combination with some polyprotic counterions, such as mono salt, and the other candidates form several combinations of salts, such as mono and hemi salts. In the crystalline form selection process, possible salts in various combinations should be prepared on a large scale and subsequently characterized because each combination of salt would show different physical properties (40). Therefore, it is important to obtain stoichiometrical information for efficient preparation of polyprotic salts.

The case that an ionic drug candidate formed only hemi salt with polyprotic counterion in salt screening has been previously reported (25). In the report, sertraline hemi-L-tartrate was obtained in 1:1 molar ratio (sertraline/L-tartaric acid); however, only polymorphism of salts was discussed, and stoichiometrical information of sertraline L-tartrate on multiwell plate was not fully elucidated (25). In this study, we performed tamoxifen salt screening on multiwell plate, and free base, counter acids, and salts could be identified by Raman microscope to obtain stoichiometrical information. Combining the information of Raman spectra of all crystals on multiwell plate, we could predict salt formation and stoichiometrical information of polybasic acid salts in addition to polymorph information. As the analytical tools for crystalline form selection, PXRD gives physical information and Raman microscopy provides both physical and chemical information. Physical information suggests the crystallinity and existence of polymorphism, whereas chemical information suggests salt formation and stoichiometrical combination of polyprotic salts. Therefore, Raman microscopy would be another useful analytical tool for salt screening, and further investigation for screening may be expected to expand.

In addition to salt screening for ionic drug candidates, cocrystals have been recently investigated as a pharmaceutical development option for neutral drug candidates (18,41). Cocrystals, categorized as multicomponent crystals such as solvate and salt, have the potential to improve drug candidate properties. Cocrystal preparations by melt crystallization, grinding, and recrystallization from solvents have been reported (11,41,42). Cocrystal screening could be performed with drug candidates and pharmaceutically acceptable excipients on multiwell plates. Cocrystal screening using Raman microscopes will also provide effective information for scaled-up preparation because there would be numerous stoichiometrical combinations with drug candidates and excipients.

In conclusion, our investigation enabled the successful development of salt screening methodology by using a Raman microscope, providing information on salt formation and stoichiometrical combinations of polyprotic salts, as well as polymorphism. Stoichiometrical information of polyprotic salts with small amount of drug candidate allows efficient crystalline form selection process by saving bulk.

ACKNOWLEDGMENTS

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