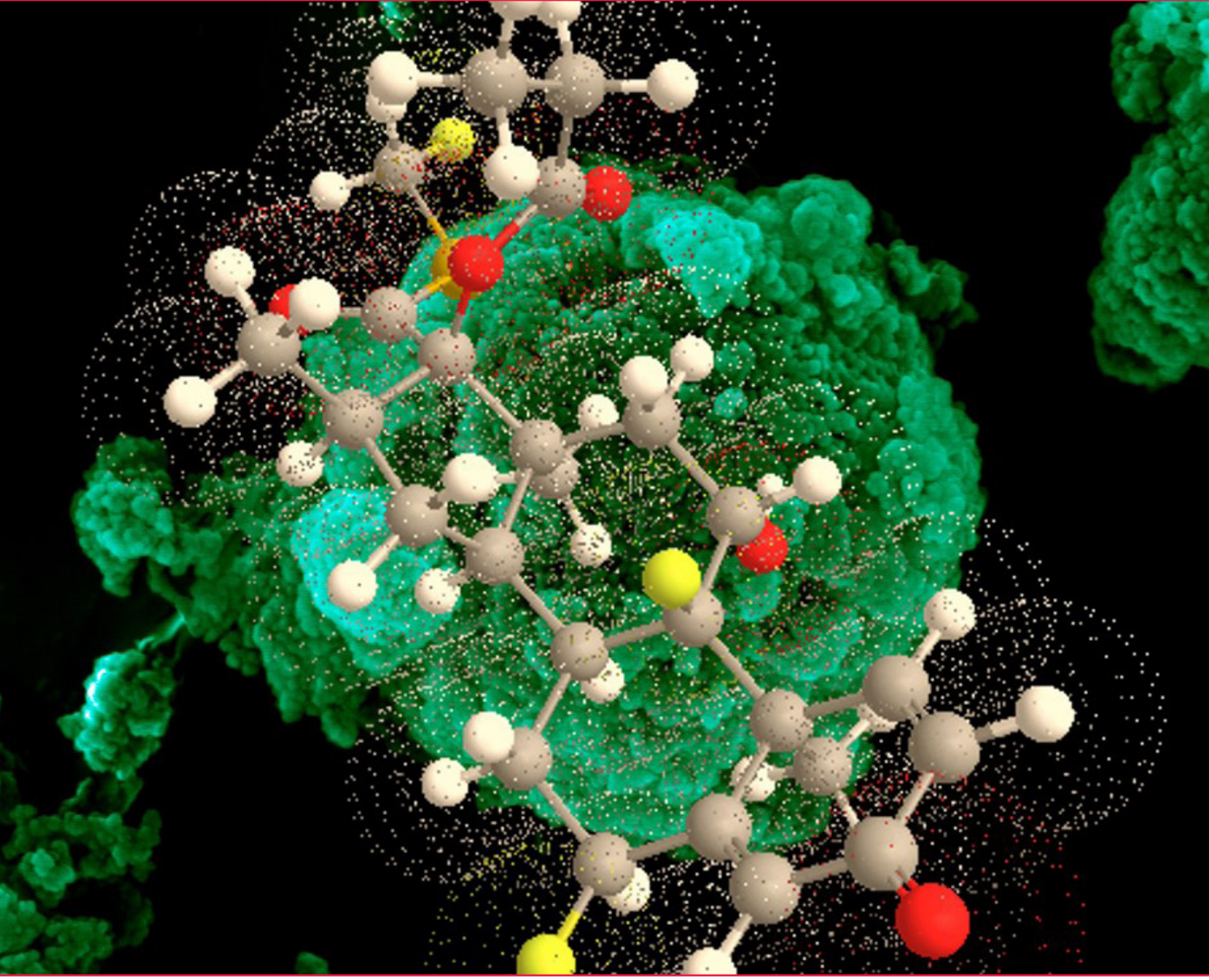

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Optimization of Monofluoromethylation Reagents: Synthesis of Pharmaceutical Steroids as a Case Study

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Abstract:

The efficient and selective incorporation of monofluoromethyl groups into organic molecules has attracted great attention in recent years. Monofluoromethylation is usually archived by direct functionalization with CH_2FBr or indirectly, *via* CH_2BrI, CH_2ClI, or similar agents. These reagents are ozone depleting substances and its use should be strictly avoided. In this work, we successfully optimized the conditions to prepare fluoromethyl phenyl sulfoxide, a key intermediate in the preparation of monofluoromethylating reagents. A protocol to perform the monofluoromethylation of two steroids was also developed, which was validate through the synthesis of two important and complex pharmaceutical drugs used in the treatment of asthma and rhinitis, fluticasone propionate and fluticasone furoate, respectively.

Keywords:

Monofluoromethylation, Fluticasone propionate, Fluticasone furoate

Introduction

The increasing interest in the fluorination chemistry, by the scientific community, is mostly a consequence of the properties that fluorine substitution can impart on organic molecules, such as in pharmaceuticals[1] and agrochemicals.[2] The high electronegativity and small size of fluorine, the replacement of hydrogen atoms by fluorine in organic compounds often results in a deep change in their physical and chemical properties, such as the stability, lipophilicity, bioavailability, metabolic stability and strength of protein-ligand binding interactions.[3] In 1970 there were only about 2% of fluorine-containing drugs on the market, while the current number has grown to about 25%. From the five top-selling pharmaceuticals three of them contain fluorine. In general, about one-third of the top-performing drugs, currently on the market, contain fluorine atoms in their structure.[4] The two major synthetic methods to prepare selectively fluorinated organic compounds are the fluorination and fluoroalkylation.[5] Although, fluorination chemistry has more than 100 years, with the first examples of nucleophilic and electrophilic fluorination reactions reported in the second half of the 19th century,[6] this chemistry still a challenge today. Fluoroalkylation chemistry, includes trifluoromethylation, difluoromethylation and monofluoromethylation. The trifluoromethylation, which consists by selective introduction of CF_3, has been extensively studied over the last four decades, including nucleophilic, electrophilic, and free radical trifluoromethylation reactions,[7] while the analogous difluoromethylation and monofluoromethylation (selective Introduction of a CF_2H or CH_2F group into organic molecules) are less studied. The systematic exploration of di- and monofluoromethylation has just emerged more recently. The interest in monofluoromethylation chemistry emerged when it was found that monofluoromethyl-containing compounds exhibit unique

biological properties.[8] As a result, a variety of structurally diverse CH_2F-containing drugs have been developed, such as fluticasone propionate **(1)**, afloqualone **(2)**[9] and fluticasone furoate **(3)**, Figure 1. Curiously fluticasone propionate is on the list of the top-selling fluorinated drugs, previously referred.

The selective incorporation of monofluoromethyl group in a molecule is usually carried out directly using CH_2FBr or indirectly, using CH_2BrI, CH_2ClI, among others. These compounds are known as hydrochlorofluorocarbons or freons (HCFCs) a subclass of chlorofluorocarbons (CFCs),[10] which are depleting compounds and therefore should be avoided for environmental reasons.

Recently, a new electrophilic monofluoromethylation reagent (S-monofluoromethyl-S-phenyl-2,3,4,5-tetramethylphenylsulfonium tetrafluoroborate)[11] **(9)** was reported for the direct transfer of a $+CH_2F$ group to nucleophiles such as sulfonic acids, tertiary amines, imidazole derivatives and phosphines. However, as shown in Scheme 1, **9** is prepared using chlorofluoromethane, an ozone depleting substance, which should be avoided.

Herein, we report a procedure to prepare **6** using alternative routes, without the use of chlorofluoromethane or other depleting reagents. We also report a protocol to carry out the monofluoromethylation of a complex compound such as steroids, fluticasone propionate **(1)** and furoate **(3)**, which are active pharmaceutical ingredients.

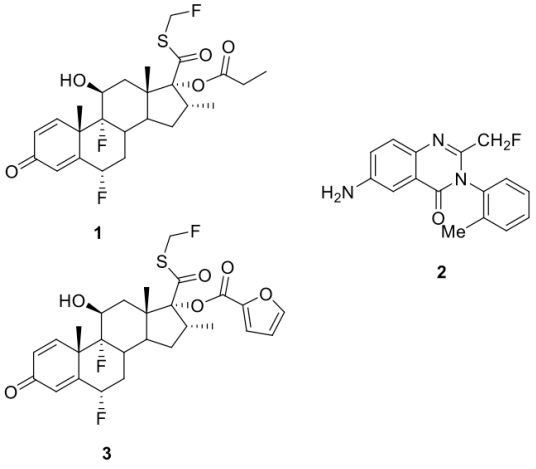
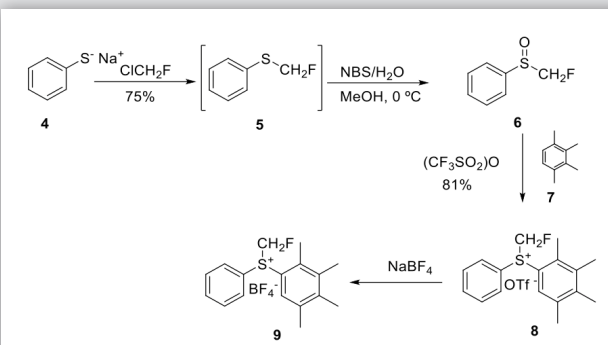


Figure 1. Examples of biological active compounds containing a monofluoromethyl group. Fluticasone propionate (1)**, Afloqualone** (2) **and Fluticasone Furoate** (3)**.**

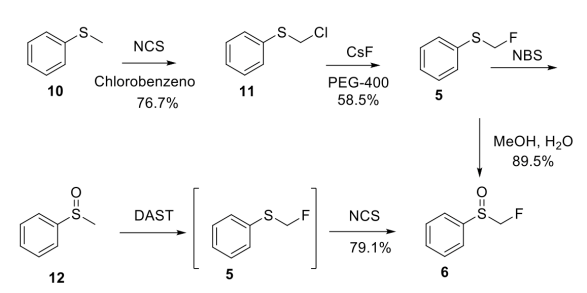


Scheme 1. Synthesis of S-monofluoromethyl-S-phenyl-2,3,4,5-tetramethylphenylsulfonium tetrafluoroborate salt (9)**.**

Results and discussion

The required fluoromethyl phenyl sulfoxide **6** is an important starting material for the stereospecific synthesis of terminal vinyl fluorides and other groups have already devised different strategies for its synthesis. So, we prepare it using two different protocols (Scheme 2), starting from methyl phenyl sulfide **(10)**, isolating each intermediate, and starting from methyl phenyl sulfoxide **(12)**, in one pot synthesis.

The production of chloromethyl phenyl sulfide **(11)** is reported to occur in high yield when N-chlorosuccinamide (NCS) is used in tetrachloromethane (99% yield)[12] or benzene (97% yield).[13] However, both solvents should be avoided due to their toxicity. So, the first challenge was to find a suitable solvent to prepare **11**. The reaction was tested in solvents such as: dichloromethane, 1,2-dichloroetane, acetonitrile and ethyl acetate. The best results were obtained in chlorobenzene at a temperature



Scheme 2. Alternative synthesis of fluoromethyl phenyl sulfoxide 6.

between 35 ºC and 45 ºC. The second challenge was the purification of this intermediate. Purification by column chromatography led to product decomposition during the elution process, but distillation (62 ºC, 40 Pascal) was found to succeed, in a scale of 100 g in good yield (76.7% yield).

Fluoromethyl phenyl sulfide **(5)** was prepared as reported,[14] starting from **11**, using cesium fluoride in a mixture of acetonitrile and PEG 200 at 80 ºC. The yield obtained (58.5%) was much lower than that reported (93%), due to the low stability of this intermediate. Nevertheless, we prepared **6** by oxidation of fluoromethyl phenyl sulphide **(5)** with *N*-bromosuccinamide (NBS) in a mixture of methanol/water at 0 5 ºC in 89.5% yield.

As mentioned before, **11** is an unstable intermediate, and for this reason we decided to prepare **6** by a different route, using a one-pot procedure. Compound **6** was successfully prepared starting from methyl phenyl sulfoxide **(12)** using the protocol of Umemoto and Tomizawa.[15] The optimized process produced product of good quality with a yield higher than reported (79.1% yield) and without using chromatography purification.[16]

Finally, the triflate salt was obtained by the Friedel-Craft reaction of compound **6** with 1,2,3,4-tetramethylbenzene **(7)** in presence of trifluoromethanesulfonic anhydride. The treatment of triflate salt solution in dichloromethane with $NaBF_4$, afforded the tetrafluoroborate salt **(9)**.

Both salts (triflate and trifluoroborate) were tested in the preparation of fluticasone propionate and furoate using different bases (cesium carbonate, potassium carbonate and sodium carbonate), at different temperatures (from room temperature to 50 ºC) and in different solvents (acetonitrile, MTBE, THF, Me-THF, heptano, DMF, 1,2-dimetoxyethane, toluene, α,α,α-trifluorotoluene, or dichloromethane). The best results were obtained in presence of cesium carbonate, due to its solubility in organic solvents, at room temperature and in dichloromethane or acetonitrile. In these conditions pure fluticasone propionate and furoate were obtained. Table 1 shows the best results obtained.

Conclusion

A novel process for the synthesis of fluoromethyl phenyl sulfoxide intermediate was disclosed. The protocol uses non-ozone-depleting reagents or toxic solvents (such as benzene or tetrachloromethane). The quality and yield obtained were excellent, without the need of chromatographic methods. This intermediate was used in the preparation of monofluoromethylating reagents (triflate and tetrafluoroborate salts) able to perform the monofluoromethylation of complex steroids in excellent yield and purity. The processes are scalable and may be applied up to an industrial scale.

Experimental section

^{1}H NMR spectra were obtained at 400 MHz in $CDCl_3$ or DMSO-d_6 with chemical shift values (δ) in ppm downfield from tetramethylsilane, ^{13}C NMR spectra were obtained at 100.61 MHz and ^{19}F NMR spectra were obtained at 376.5 MHz. Assignments are supported by 2D correlation NMR studies. Some reactions were monitored by Waters High Performance Liquid Chromatographer (HPLC) model 600, equipped with auto sampler w717 plus and Photo Didode Array (PDA) detector W996. Medium pressure preparative column chromatography: Silica Gel Merck 60 H. Analytical TLC: Aluminium-backed Silica Gel Merck 60 F254. Reagents and solvents were purified and dried according to Purification of Laboratory Chemicals book.[17]

Preparation of monofluoromethyl phenyl sulfoxide (6) starting from mthylphenylsulfide (10)

Each intermediate was isolated.

Preparation of chloromethyl phenyl sulfide (11)

Methyl phenyl sulfide (100 g, 805.15 mmol) was diluted in chlorobenzene (602 mL). N-Chloro Succinimide (NCS) (112.89 g, 1.05 eq) was added in small portions maintaining the temperature between 35 ºC and 45 ºC, under an argon

Table 1. Synthesis of fluticasone propionate and furoate

Final Product	Sulfonium salt	Cs_2CO_3 (eq)	Solvent	Purity (% area by HPLC)	Molar yield (%)
Fluticasone propionate	**8** (1.00 eq)	1	CH_3CN (10 vol)	96.23	92.9
Fluticasone propionate	**9** (1.18 eq)	1	CH_2Cl_2 (10 vol)	99.73	84.2
Fluticasone furoate	**8** (1.43 eq)	0.65	CH_3CN (4 vol)	99.41	88.4
Fluticasone furoate	**9** (1.18 eq)	0.65	CH_3CN (4 vol)	99.64	88.4

atmosphere. After 3 hours, the suspension formed was filtered, and the solid was washed with chlorobenzene (50 mL). The filtrate was washed with water (3x 300 mL). The resulting organic phase was dried with magnesium sulfate and concentrated. The crude product was purified by distillation to give 98 g (76.7%) of the desired product as yellow oil (bp: 62 ºC at 40 Pa).

Preparation of fluoromethyl phenyl sulfide (5)

Cesium fluoride (191.50 g, 2 eq) was added to a mixture of PEG400 (100 mL) and acetonitrile (600 mL). The mixture was stirred for a few minutes under an argon atmosphere and then acetonitrile (100 mL) was removed by distillation. Chloromethyl phenyl sulfide (100 g, 630.35 mmol) was added and the resulting mixture was stirred for 6 hours at a temperature between 80 ºC and 85 ºC. The mixture was filtered and the filtrate was concentrated. The crude product was purified by distillation to give 52.46 g (58.5%) of the desired product as slightly yellow oil (bp: 43 ºC at 40 Pa).

Preparation of fluoromethyl phenyl sulfoxide (6)

Fluoromethyl phenyl sulphide (50 g, 351.64 mmol) was added to a mixture of methanol 250 mL and water (50 mL). The resulting mixture was cooled to a temperature between 0 ºC and 5 ºC. NBS (75.10 g, 1.2 eq) was added in small portions maintaining the same temperature range. The reaction mixture was stirred until the reaction was complete, and then, was quenched with Na_2SO_3 solution (10%, 150 mL). The pH of the reaction mixture was adjusted to a value between 7 and 8 with $NaHCO_3$ saturated solution. The mixture was concentrated under vacuum at a temperature between 20 ºC and 25 ºC. The residue was extracted with dichloromethane (200 mL, 300 mL). The combined organic layer was washed with water (2x 300 mL) and concentrated to 1/3 of the volume. Heptane (50mL) was added and the resulting mixture was concentrated again. The crude product was purified by flash chromatography (Ethyl Acetate/Hexane 30:70) to give 49.8 g (89.5%) of the desired product as colourless oil at rt, which is a white solid at -20 ºC. The spectral data of fluoromethyl phenyl sulfoxide is in good agreement with the reported data.[11]

^{1}H NMR ($CDCl_3$), 400 MHz: 7.70-7.68 (m, 2H), 7.59-7.57 (m, 3H), 5.15 (d, 1H, *J*H-F = 2.6 Hz), 5.03 (d, 1H, *J*H-F = 48.08 Hz).

One-pot synthesis of monofluoromethyl phenyl sulfoxide ***(6)*** *starting from methylphenylsulfoxide* ***(12)***

Methylphenylsulfoxide (65 g, 463.62 mmol) was dissolved in dichloromethane (500 mL) under nitrogen atmosphere. The solution was cooled to a temperature below -5 ºC. DAST (100 mL, 1.64 eq) was added slowly maintaining the same temperature. The reaction mixture was warmed up to room temperature and stirred for 1 hour at this temperature and then, overnight at the same temperature. Water (300 mL) was added after cooling the mixture to 0 ºC and then the reaction mixture was warmed up until a temperature between 20 ºC and 25 ºC. The resulting mixture was stirred, and the layers were separated. The aqueous phase was extracted with dichloromethane (3x 400 mL). The combined organic phase was washed with saturated $NaHCO_3$ (400 mL) and saturated NaCl solution (400 mL) and then was concentrated to dryness, an oily residue was obtained. The residue was cooled to 0 °C and dissolved in a mixture of methanol (297.1 mL) and water (59.4 mL). NBS (N-bromo Succinimide (100.5 g, 1.22 eq) was added in small portions and the resulting solution was stirred at the same temperature until the reaction was complete. The mixture was quenched with the addition of Na_2SO_3 solution (10%, 300 mL). Saturated $NaHCO_3$ solution was added to adjust the pH between 7 and 8 and then the mixture was concentrated under vacuum at a temperature between 30 ºC and 35 ºC. The residue was extracted with dichloromethane (3x 300 mL). The combined organic phase was dried with anhydrous sodium sulfate and then concentrated under vacuum to give the crude product as yellow oil, 58 g (79.1%). The spectral data of fluoromethyl phenyl sulfoxide is in good agreement with the reported data.[11]

Preparation of S-monofluoromethyl-S-phenyl-2,3,4,5-tetramethylphenylsulfonium triflate salt ***(8)***

To a solution of monofluoromethyl phenyl sulfoxide (50 g; 316.07 mmol) in diethyl ether (550 mL) was added 1,2,3,4-tetramethelbenzene (47,14 mL; 1,0 eq) and the resulting mixture was cooled to a temperature lower than -5 ºC. Trifluoromethanesulfonic anhydride was added (55.73 mL; 1.05 eq) maintaining the same temperature. The mixture was stirred until the reaction was complete. The precipitate triflate salt formed was isolated by filtration, washed with diethyl ether at 0 ºC and dried. A white solid was obtained (132 g) with 99.98% (% area by HPLC) purity

and 98.4% yield. The spectral data of S-monofluoromethyl-S-phenyl-2,3,4,5-tetramethylphenylsulfonium triflate salt is in good agreement with the reported data.[11]

^{1}H NMR ($CDCl_3$), 400 MHz: d 7.77-7.64 (5H, m), 7.42 (1H, s), 6.64 (1H, dd, J=42.2 Hz, J=9.5 Hz), 6.52 (1H, dd, J=41.1 Hz, J=9.5 Hz), 2.50 (3H, s), 2.38 (3H, s), 2.31 (3H, s), 2.29 (3H, s).

Preparation of S-monofluoromethyl-S-phenyl-2,3,4,5-tetramethylphenyl sulfonium tetrafluoroborate salt (9)

The triflate salt was (10 g; 23.6 mmol) was dissolved in dichloromethane (80 mL). The mixture was washed with $NaBF_4$ (1M, 5x 100 mL). The combined organic phases were dried with magnesium sulfate. The solvent was removed by evaporation under vacuum. A white solid was obtained (7.4 g) with 99.82% (% area by HPLC) purity and 86.7% yield. The spectral data of S-monofluoromethyl-S-phenyl-2,3,4,5-tetramethylphenylsulfonium tetrafluoroborate salt are in good agreement with the reported data.[11]

^{1}H NMR ($CDCl_3$), 400 MHz: d 7.79-7.43 (5H, m), 7.43 (1H, s), 6.56 (1H, dd, J=28 Hz, J=9.5 Hz), 6.45 (1H, dd, J=27 Hz, J=9 Hz), 2.49 (3H, s), 2.38 (3H, s), 2.30 (3H, s), 2.29 (3H, s).

^{13}C NMR (CDCl3), 100 MHz: d 143.9, 139.4, 138.2, 137.5, 134.3, 131.3, 130.8, 128.4, 121.2, 116.2, 89.6 (d, J=240.3 Hz), 21.1, 17.7, 16.9, 16.8.

Preparation of fluticasone propionate (1)

With S-monofluoromethyl-S-phenyl-2,3,4,5-tetramethylphenylsulfonium triflate (8) in acetonitrile

17- Propionate carbothioic acid (5 g, 10.7 mmol) was suspended in acetonitrile (50 mL). Cesium carbonate (3.39 g, 1 eq) was added and the resulting suspension was stirred for 5 minutes at room temperature. S-monofluoromethyl-S-phenyl-2,3,4,5-tetramethylphenylsulfonium triflate (4.54 g, 1 eq) was added. The suspension was stirred at room temperature until the reaction was complete. The solid was isolated by filtration, washed with acetonitrile (10 mL) and then with heptane (2x 10 mL) at 5 ºC. The solid was dried under vacuum at a temperature below 35 ºC. The solid obtained was recrystallized from a mixture of acetone and water. A white solid was obtained with 96.23% (% area) purity by HPLC and 92.9% yield. The salts are purged during this recrystallization.[16]

With S-monofluoromethyl-S-phenyl-2,3,4,5-tetramethylphenyl sulfonium tetrafluoroborate (9) in dichloromethane

17- Propionate carbothioic acid (5 g, 10.7 mmol) of was dissolved in dichloromethane (50 mL). Cesium carbonate (3.39 g, 1 eq) of was added and the solution turned into a suspension. The suspension was stirred for 40 minutes at room temperature. S-monofluoromethyl-S-phenyl-2,3,4,5-tetramethylphenyl sulfonium tetrafluoroborate (4.57 g, 1.18 eq) was added. The mixture was stirred at room temperature until the reaction was complete. The solid was isolated by filtration, washed with dichloromethane (10 mL) and then with heptane (2x 10 mL). The solid was dried under vacuum at a temperature below 35 ºC and then recrystallized from a mixture of acetone and water. The product was obtained with 99.73% (% area) purity by HPLC and 84.2% yield. The salts are purged during this recrystallization.[16]

Preparation of fluticasone furoate (3)

With N-(monofluoromethyl)-N-phenyl-dimethylammonium triflate (8) in acetonitrile in acetonitrile

Carbothioic acid furoate (2.5 g, 4.93 mmol) was suspended in acetonitrile (10 mL). Cesium carbonate (1.04 g, 0.65 eq) and N-(monofluoromethyl)-N-phenyl-dimethylammonium triflate (3.0 g, 1.43 eq) were added and the suspension was stirred for 4 hours at room temperature. The solid was isolated by filtration, washed twice with acetonitrile (2.5 mL) previously cooled to 5 ºC and dried under vacuum at a temperature below 35 ºC. The solid obtained was recrystallized from a mixture of acetone and water. A white solid was obtained with 99.41% (% area) purity by HPLC and 88.4% yield. The salts are purged during this recrystallization.[16]

With S-monofluoromethyl-S-phenyl-2,3,4,5-tetramethylphenylsulfonium tetrafluoroborate (9) in acetonitrile

Carbothioic acid furoate (2.5 g, 4.93 mmol) was suspended in acetonitrile (10 mL). Cesium carbonate (1.04 g, 0.65 eq) of S-monofluoromethyl-S-phenyl-2,3,4,5-tetramethylphenylsulfonium tetrafluoroborate (2.1 g, 1.18 eq) were added and the suspension was stirred for 1 hour at room temperature. The solid was isolated by filtration,

washed twice with acetonitrile (2.5 mL) previously cooled to 5 ºC, and dried under vacuum at a temperature below 35 ºC. The solid obtained was recrystallized from a mixture of acetone and water. A white solid was obtained with 99.64% (% area) purity by HPLC and 88.4% yield. The salts are purged during this recrystallization.[16]

Acknowledgments

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Byproducts of Commonly Used Coupling Reagents:

Origin, Toxicological Evaluation and Methods for Determination

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Genentech Research and Early Development
Genentech (a member of the Roche Group)

Introduction

Ensuring the purity and high quality of drug substances is a critical aspect of drug development and patient safety. Organic impurities are described in international guidelines.[1–3] However, the guidelines are focused on the identification, qualification and specifications of impurities which are structurally related to the drug substance, or residual solvents and catalysts. Because amide and ester functional groups are ubiquitous in drug substances, we decided to address the qualification of byproducts formed from common coupling reagents used to prepare these common functional groups. In general, coupling reagents act in a similar manner by first activation of the carboxylic acid group; followed by nucleophilic attack of the amine or alcohol to form the corresponding amide or ester[4] functional group. The general scheme as shown in Figure 1 illustrates the basic amide bond forming reaction by treatment of a carboxylic acid with a coupling reagent (CR), leading to the activated complex, which is then treated with a nucleophilic amine source to form the amide bond and the coupling reagent byproduct(s).

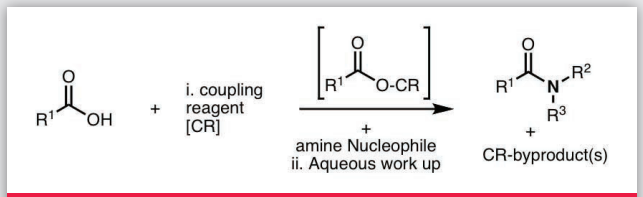


Figure 1. Schematic illustrating the generation of an amide bond in the final product and a byproduct using a coupling reagent (CR).

Selection of an effective coupling reagent is typically based on functional group selectivity, yield, and minimization of side-reactions after a screen of the readily available reagents. However, consideration of organic reaction byproducts from these reagents, which are not structurally related to the drug substance (DS), is often disregarded during development. These byproducts are often not subjected to the identification and qualification thresholds described in ICH Q3A and B[1,2] nor are they treated the same way as residual solvents as described in ICH Q3C.[3] The specifications of these byproduct impurities can be considered individually, based on the available toxicological data, daily dose, duration of therapy, and other risk-benefit considerations. For several commonly-used coupling reagents utilized in large-scale drug manufacturing, the relevant impurities and toxicological data are summarized in Table 1. In several instances, byproducts from the coupling reagent can be formed and the known ones are listed in Table 1 after an aqueous work-up.

A search of the toxicology databases (e.g., Hazardous Substances Data Bank (HSDB),[5] Registry of Toxic Effects of Chemical Substances (RTECS)[6]) was conducted for each of the byproducts listed in Table 1. In most cases, toxicological data were not available. Subsequently, these substances were evaluated using industry standard *in silico* structure-activity relationship models, SAR/(Q)SAR, (i.e. DEREK [Lhasa Ltd; Leadscope {Leadscope, Inc]) to predict potential mutagenic and/or carcinogenic activity as well

Table 1. Common coupling reagents, byproducts and proposed TTC for critical process parameters.

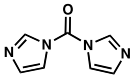
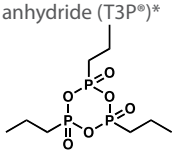
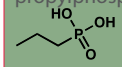
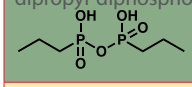
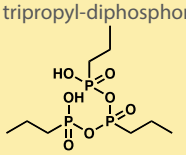
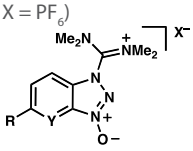
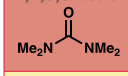
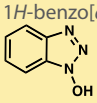
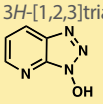
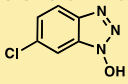
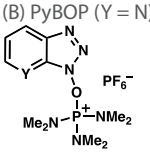
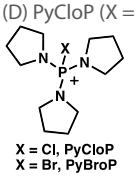
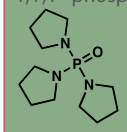
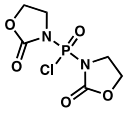
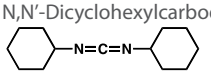
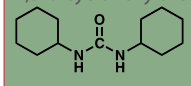
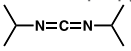
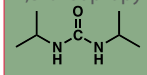
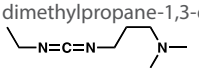
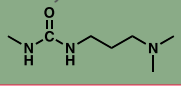
Coupling Reagent	CR-Byproduct	*In Silico* Evaluation and Summary of Toxicological Data	Proposed TTC µg/day
1,1-carbonyldiimidazole (CDI) 	Imidazole 	No structural alerts; Reproductive/developmental toxicity at high dose	100
Propylphosphonic anhydride (T3P®)* 	propylphosphonic acid 	No structural alerts; No reported toxicological data	100
	dipropyl-diphosphonic acid 	No structural alerts; No reported toxicological data	100
	tripropyl-diphosphonic acid 	No structural alerts, Skin and eye irritant	10
Uronium coupling reagents: (A) HBTU (Y = C; R = H; X = PF_6) (B) HCTU (Y = C; R = Cl; X = PF_6) (C) HATU (Y = N; R = H; X = PF_6) 	1,1,3,3-tetramethyl-urea (TMU) 	Reproductive/developmental toxicity	1
	1*H*-benzo[*d*][1,2,3]-triazol-1-ol (HOBt) 	No structural alerts, Skin and eye irritant	10
	3*H*-[1,2,3]triazolo-[4,5 *b*]pyridin-3-ol (HOAt) 	No structural alerts, Skin and eye irritant	10
	6-chloro-1*H*-benzo-[*d*][1,2,3]triazol-1-ol 	Structural similarity to HOBt and HOAt	10
	Hexafluoro Phosphate ion 	No structural alerts; No reported toxicological data	100

Table 1. Common coupling reagents, byproducts and proposed TTC for critical process parameters. (con'd.)

Coupling Reagent	CR-Byproduct	In Silico Evaluation and Summary of Toxicological Data	Proposed TTC µg/day
Phosphonium coupling reagents: (A) BOP (Y = C) (B) PyBOP (Y = N) 	Hexamethyl-phosphoramide (HMPA)  [1*H*-benzo[*d*][1,2,3]-triazol-1-ol (HOBt) and **3*H*-[1,2,3]triazolo-[4,5 *b*] pyridin-3-ol (HOAt)** see Uronium Coupling Reagents]	Likely human carcinogen	1
(C) PyCloP (X = Cl) (D) PyCloP (X = Br)  X = Cl, PyCloP X = Br, PyBroP	1,1′,1″-phosphoryl-tripyrrolidine 	No structural alerts; No reported toxicological data	100
(E) BOP–Cl 	oxazolidin-2-one 	No structural alerts; No reported toxicological data	100
Carbodiimide coupling reagents: N,N′-Dicyclohexylcarbodiimide (DCC)  Guinea Pig: LD50 = 10 mL/kg (Skin)	1,3-dicyclohexylurea 	No structural alerts; No reported toxicological data	100
N,N′-Diisopropylcarbodiimide (DIC)  Rat: LC50 = 0.41–0.922 mg/L (6h) Rat: LC50 = 20 ppm/4h	1,3-diisopropylurea 	No structural alerts; No reported toxicological data	100
N^1-((ethylimino)methylene)-N^3,N^3-dimethylpropane-1,3-diimide (EDC) 	1-(3-(dimethylamino)-propyl)-3-methylurea 	No structural alerts; No reported toxicological data	100

* T3P® registered trademark of Clariant

as other toxicologic end points. The results from both evaluations were combined and a Threshold of Toxicologic Concern, TTC, assigned based on the approach described by Dolan *et al.*[7] Briefly, the TTC principle is a level of human exposure which is estimated to pose no appreciable risk to human health for a lifetime exposure. Dolan et al derived the TTCs by analyzing the available data for regulated carcinogens and noncarcinogens to provide a scientific rationale for recommendations of acceptable limits for three classes of compounds with little or no toxicity data: (1) compounds that are likely to be carcinogenic, (2) compounds that are likely to be potent or highly toxic, and (3) compounds that are not likely to be potent, highly toxic or carcinogenic. Corresponding TTCs for these categories of materials are 1, 10 and 100 µg/day, respectively. These categories address all types of toxicological endpoints, including carcinogenicity, immunotoxicity, neurotoxicity, and developmental toxicity. The thresholds for these categories are based on the assumption that, even if subsequent testing were to indicate that, in this case the coupling agent, were to fall into one of these three categories, exposures below the TTC level pose no appreciable risk to human health. CR-byproducts in Table 1 are color coded red, yellow and green based on their designation as a Class 1, 2 or 3 compounds.

Discussion

Carbodiimide Coupling Reagents

Chemistry

Carbodiimides were the first coupling reagents to be synthesized and are still widely used. The first step in coupling involves the reaction of the carboxylic acid with the carbodiimide to form the O-acylurea. Problems with epimerization and yield have led to the development of additives such as hydroxy-benzotriazole (HOBt)[4] and other coupling reagents. The carbodiimide and resulting urea byproducts have been designed as either water soluble (e.g. EDC/ethyl-(N',N'-dimethylamino) propyl urea) or water insoluble (e.g. DCC/dicyclohexyl urea) which influence the selection of reaction solvents and purification strategy.

Analysis

As a coupling reagent with a long history of use, methods of detection and analysis have been investigated using differing strategies from colorimetric detection to separations. Early studies were limited by the poor UV absorption focused on colorimetric analysis. Utilizing the reactivity of carbodiimides, several colorimetric assays for their detection have been developed.[8,9] In one assay, pyridine and barbituric acid are reagents that form a brightly colored reaction product in the presence of carbodiimides. The reaction scheme is shown in Figure 2. Colorimetric assays have been shown to work with common coupling carbodiimide containing reagents such as EDC, DCC, 1-cyclohexyl-3(2-morpholinoehtyl)-carbodiimide metho-p-toluene sulfonate (CMCT) and DIC. The reaction products are detected at their maximum absorbance of 595nm. The major limitation of the colorimetric assay is that it can only detect unreacted carbodiimides while the urea byproducts are not detected. This limitation has led to the development of more modern LC-MS methodologies which used mass spectrometry for detection to overcome the poor UV absorption.[10]

LC-MS and LC-MS/MS approaches to the detection of carbodiimides are gaining popularity due to their specificity and sensitivity.[11] Typically, these methods are set up as limit tests to demonstrate that the EDC and its urea byproduct have been sufficiently purged from the process. Due to the rapid reactivity of EDC during sample preparation and analysis, EDC and its corresponding urea byproduct are generally summed to yield the total carbodiimide. Figure 3 shows representative chromatograms for EDC and its urea byproduct. This method demonstrated the ability to separate and detect EDC and the urea byproduct.

Instrument: UHPLC with single quadrupole MS. Column: C18; mobile phase, isocratic 98% 200mM Ammonium Formate pH 4 and 2% Acetonitrile ; column temperature 10°C; Detection, SIM mode, EDC at 156 amu and urea byproduct at 174 amu. Note: The EDC and urea signals were normalized to appear on the same scale

Onium Coupling Reagents

Chemistry

Several coupling reagents are based on the HOBt/substituted HOBt systems and onium salts. These reagents react with carboxylic acids to form active esters, which then react with amines.[4] A side-reaction can often take place where the

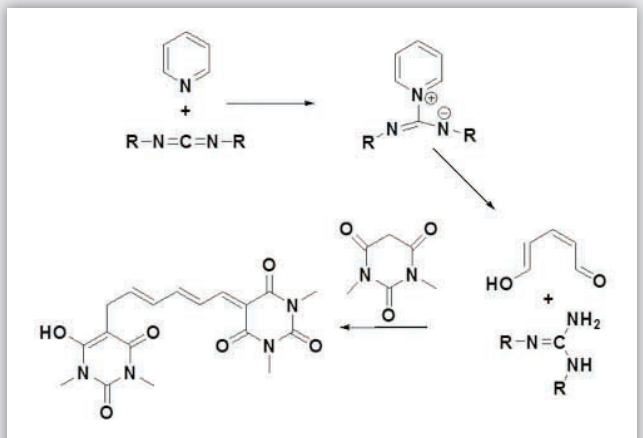


Figure 2. Reaction Scheme for Colorimetric Analysis of Carbodiimides

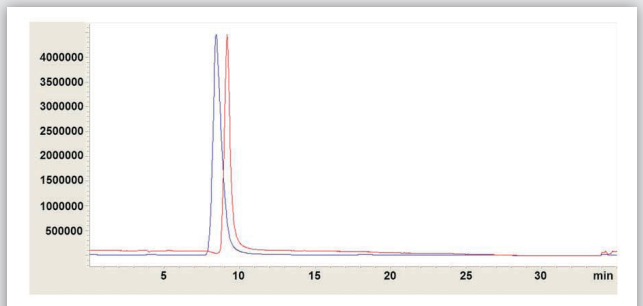


Figure 3. Representative Chromatograms of EDC (red) and its Urea Byproduct (blue) by Reversed Phase LC-MS with Single Ion Monitoring (SIM) Signals

amine reacts with the coupling reagent to form a guanidinium byproduct, thus order of addition and timing are crucial. Reactions are generally rapid with little epimerization.

Analysis

Methods to determine residual Hydroxyl-benzotriazole (HOBt), tetramethylurea (TMU) and PF_6- in API were developed in our laboratories. HOBt was determined using Reversed Phase HPLC with UV detection; PF_6- was determined using Reversed Phase HPLC with CAD (Charged Aerosol Detection).TMU was determined with Head Space-GC using FID Detection. The method development was challenging due to the range of products involved and poor UV absorption. Representative chromatograms with chromatographic conditions are presented in: Figure 4 for HOBt, Figure 5 for PF_6- determination and Figure 6 for TMU with chromatographic conditions summarized in Table 2.

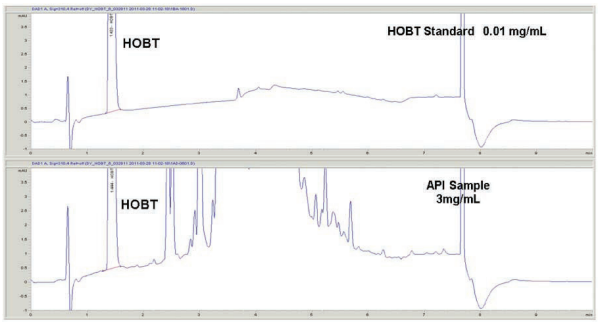


Figure4. Representative Chromatogram of HOBt by Reversed Phase HPLC with UV Detection

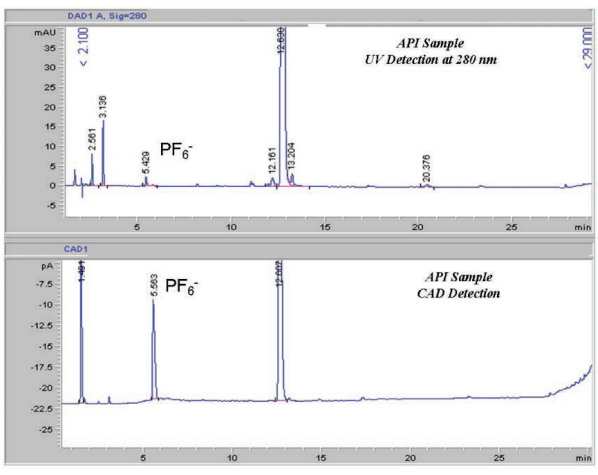


Figure 5. Representative Chromatogram of PF_6- by Reversed Phase HPLC with CAD and UV Detection Note that PF6- is not detected by UV.

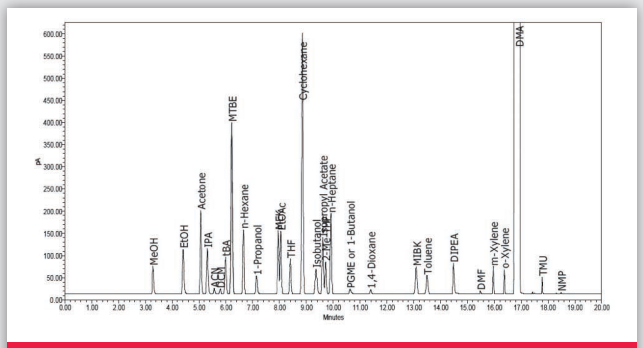


Figure 6. Representative Chromatogram of TMU by Head Space-GC with FID Detection [12] Showing Other Common Solvents in a Generic Method for Residual Solvent Analysis

Table 2. GC-Headspace Parameters for Determination of [12].

Parameter	Setting		
Column	Agilent J&W DB-624, 30 m x 0.32 mm, 1.8 µm, or equivalent		
Inlet temperature, liner	225 °C, 2-mm deactivated liner		
Split ratio	5:1		
Column flow	Helium at 1.5 mL/min (constant flow)		
Oven temperature	40 °C		
Oven temperature program	Ramp	Hold Time	Final Temp
	NA	4 min	40 °C
	8 °C/min	0 min	60 °C
	5 °C/min	2 min	85 °C
	30 °C/min	2 min	220 °C
Total run time	20.0 min		
Detector temperature	FID, 270 °C		
Detector gas flow	Hydrogen Air Makeup (helium)		40 mL/min 400 mL/min 30mL/min
Headspace autosampler	temperature: Oven, loop, transfer line Time (min): vial equilibration, pressurization, loop fill, loop equilibration Pressure: vial and transfer line Loop volume Inject time Vial shaking GC cycle time		100, 110, 150 °C 10.0, 0.2 min 0.2, 0.05 min 15, 25 psi 1.0 mL 1.0 min High 25 min

Column: C18 3um 100x3.0 mm; Mobile Phase: A 20 mM ammonium formate pH 3.7, B Acetonitrile; Gradient: 10 to 30% in 3 min, 30 to 100% B in 3 min, hold at 100% B for 1 minute; Column Temp: 40 °C; Flow rate: 1.0 mL/min; UV Detector: 310 nm; Sample Concentration: 3 mg/mL of API in 50:50 ACN/water; Injection Volume: 20 µL

Column: C18 3 µm 150 x 4.6 mm; Mobile Phase A: 20 mM ammonium formate pH 3.7, Mobile Phase B: 0.05% formic acid in Acetonitrile; Gradient: 15 to 40% B in 25 min, 40 to 90% B in 3 min; Column Temp: 30 oC; Flow rate: 1.0 mL/min; Inj Vol: 5 µL; UV Detector: 280 nm and CAD; Sample Concentration: 1.0 mg/mL of API in 50:50 ACN/water; Injection Volume: 10 µL

Other Coupling Reagents

1,1'-carbonyldiimidazole (CDI)

Chemistry

The search for coupling reagents better than carbodiimides has led to the development of CDI (1,1'-carbonyldiimidazole) and related carbonylimidazoles.[12] For practical considerations, it should be noted that moisture must be carefully excluded during work with CDI and that stoichiometric excess should be avoided.

Analysis

A method to determine residual imidazole in API was developed using mixed-mode HPLC. The method development was challenging due to the relatively low molecular weight and high polarity. Method validation is summarized in Table 3 and a representative chromatogram with chromatographic conditions is presented in Figure 7.

Column: Mixed mode embeded acetic ion-pairing, 2x50 mm, 5 µm; Mobile Phase A: 0.05% trifluoroacetic acid (TFA) in water, Mobile Phase B: 0.05% TFA in Acetonitrile; Gradient: Hold at 50% B for 1.5 min, 50-80% in 1.5 min, hold at 80% for 0.5 min; Column Temp: 35 °C; Flow rate: 1.0 mL/min; UV Detector: 205 nm; Sample Concentration: 10.0 mg/mL of API in 50:50 ACN/water; Injection Volume: 10 µL.

Table3. Method Validation Data Summary for Residual Imidazole in API

Parameter	Level	Results
Sensitivity	Limit of Quantitation Limit of Detection	0.05% 0.02%
Linearity	0.05% to 0.75%	Slope: 689.2 Y-intercept: 0.0 R_2 = 1.00 R= 1.00
Accuracy and Repeatability	0.25% 0.50% 0.75%	Mean (n=3) 99.0% % RSD 1.6% Mean (n=3) 100.3% % RSD 0.2% Mean (n=3) 99.9% % RSD 1.0%

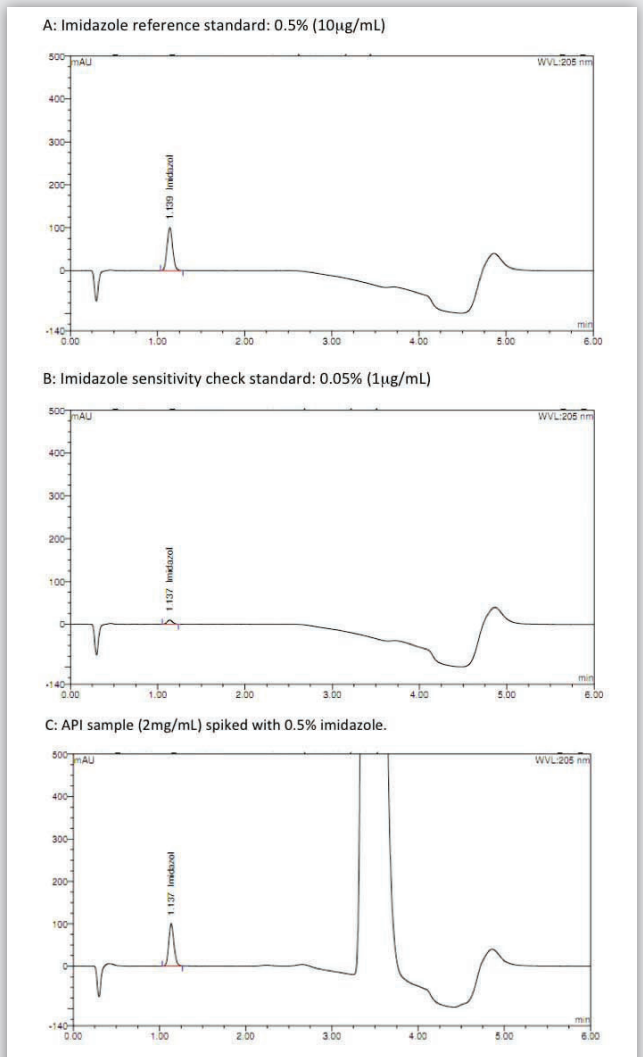


Figure 7. Representative Chromatogram of Imidazole by Mixed Mode HPLC with UV Detection

Propylphosphonic Anhydride (T3P)

Chemistry

T3P Coupling agent is used due to the high yields with low epimerization. They pose little health or environmental risk, and the resulting byproducts allow for simple phase extraction. T3P converts the oxygen of a carboxylic acid into an ionic leaving group, which is extracted from the product. T3P compares favorably to other coupling agents with respect to: yields, epimerization rates, overall process costs, and safety/toxicity.[13]

Analysis

An LC-MS (negative ionization mode) method to determine residual Propylphosphonic acids in API was developed

using Reversed-Phase HPLC. The sensitive detection of the analytes required LC-MS due to their relatively low UV absorbance. Although the method is capable of resolving mono, di and tri propylphosphonic acids; the predominant residual product was found to be the mono acid. A representative chromatogram with chromatographic conditions is presented in Figure 8.

Column: C18, 150 mm x 4.6 mm, 3 µm; Mobile Phase A: 20 mM Ammonium Formate buffer, pH 3.7, Mobile Phase B: 0.05% Formic acid in Acetonitrile; Gradient: 98-5% Bottle A in 8 min, hold at 5% for 2 min; Column Temp: 30 °C; Flow rate: 0.8 mL/min; Inj Vol: 20 mL; Sample Concentration: 1.0 mg/mL of API in 50:50 Mobile Phase A/Mobile Phase B; Injection Volume: 10 µL; MS Detection: Source: ESI, Mode: Single Ion Monitoring (SIM Ion 335.10, 229.10 and 123.00), Polarity: Negative.

Conclusion

Coupling reagents are widely used to form amides and esters for the synthesis of active pharmaceutical ingredients (API). Consideration of the reaction byproducts of these reagents are often disregarded during process development since the byproducts are not structurally related to the drug substance. This paper discusses the origin, toxicological evaluation and testing methods for reaction byproducts from many commonly-used coupling reagents including: carbodiimides, aminium-based, phosphorous-based reagents as well as simple coupling reagents such as 1,1-carbonyldiimidazole (CDI). This information should prove useful to others facing similar challenges for assessment and control of these byproduct impurities.

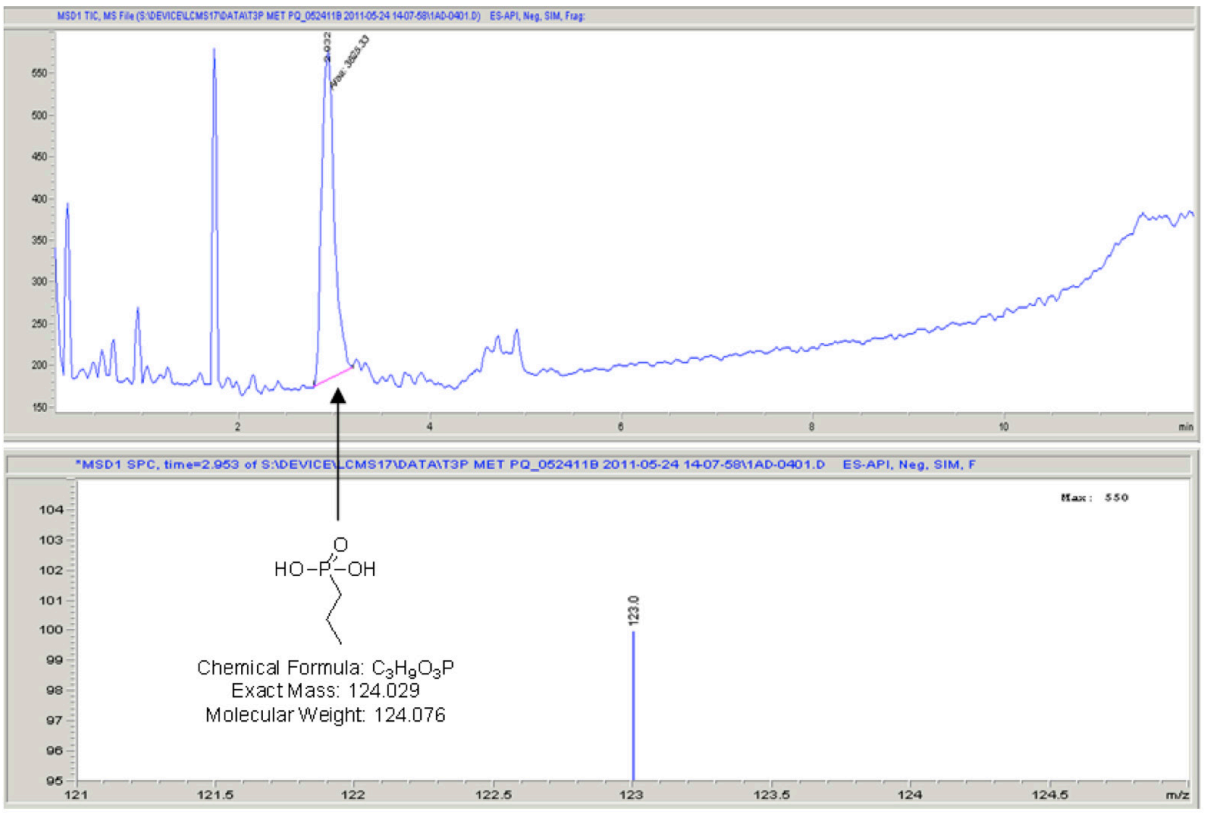
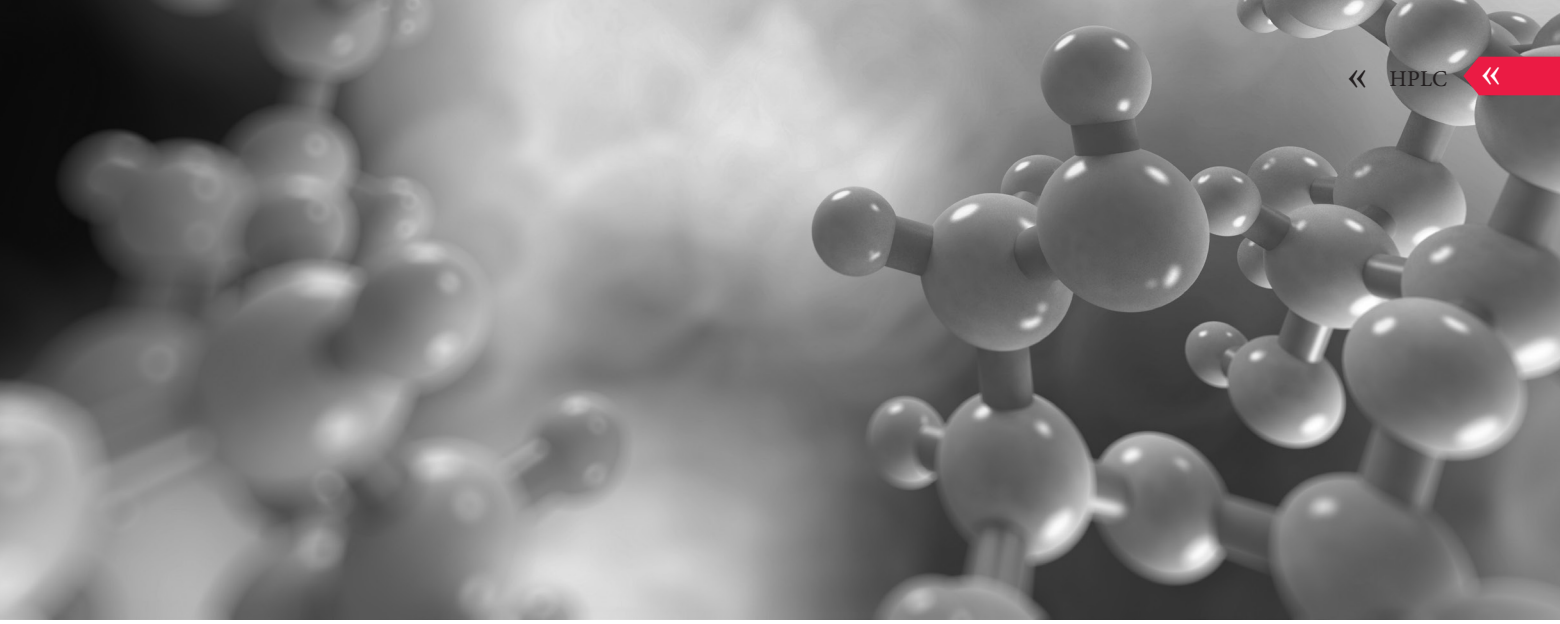


Figure 8. Representative Chromatogram of Propyl Phosphoric Acids by Reversed Phase LC-MS with Total Ion Current (TIC) Signal and Corresponding Mass Spectrum



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Analytical Methodology for Characterization of Reactive Starting Materials and Intermediates Commonly Used in the Synthesis of Small Molecule Pharmaceuticals

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Abstract

This review focuses on the analytical challenges of chromatographically characterizing sulfonate salts/esters, hydrazine functionalities, amines, boronate esters/acids, aldehydes, and sulfonate acid/esters, and acyl (acid) halides used in the synthesis of pharmaceutical drug substances. Special focus is placed on stability, degradation, and achieving the low level sensitivity required for genotoxic impurity analysis. Final pharmaceutical drug substances are designed to be stable to meet shelf life requirements, survive pharmaceutical processing into drug products, and intact delivery through the GI and/or bloodstream to their sites of action. However, the building blocks of these APIs are not constrained by the same stability requirements and may require special considerations to be accurately analyzed.

Introduction

Reactive molecules are the building blocks of synthetic organic chemistry and can be found throughout the pharmaceutical industry in Suzuki Couplings, antibody drug conjugations, etc. Their reactive nature drives syntheses, thereby allowing the catalysts, reagents, and reaction conditions to focus on the selectivity of the reaction. Even in the coupling of two non-reactive molecules, one of the components will often be converted to a reactive species *in situ* prior to formation of the bond between the two molecules. In the pharmaceutical industry, reactive molecules are often utilized as starting materials and isolated intermediates in the synthesis of the complex, selective, and biologically active new small molecule pharmaceuticals. As commercial processes are developed, reactive molecules are often shifted from starting materials and isolated intermediates to un-isolated intermediates; however, the control of these un-isolated intermediates remains critical to the robustness, cost, quality, safety and environmental impact of the synthetic route.[1] Additionally, some molecules that are deemed unreactive from a synthesis perspective can be considered reactive in the analytical laboratory due to the necessary exposure to water and/or air during sample preparation and analysis.

Accurate analysis of these reactive molecules is key to developing, monitoring, and controlling pharmaceutical syntheses from early development through commercial manufacturing. The data obtained from these analyses are used to set purchasing specifications, ensure proper charging for reaction stoichiometry, monitor the progress of the reaction, study yield and mass balance of processes in designed experiments, and evaluate purity of the reactive products.

Accurate and sensitive analysis of reactive molecules poses a challenge when decomposition is encountered during sample preparation and testing. These molecules can

decompose by various mechanisms including: oxidation, reduction, hydrolysis, polymerization, condensation, elimination, substitutions and isomerization.[2,3] Further, the inherent reactivity of these molecules often raises concerns regarding their reaction with DNA and consequently concerns of mutagenic potential. This mutagenic potential is of increasing concern to health authorities often requiring control of potentially mutagenic compounds to ppm/ppb levels.[4,5]

Direct spectral analysis by techniques such as quantitative NMR (nuclear magnetic resonance)[6] and vibrational spectroscopy (mid-IR, near-IR and Raman)[7] is often used to minimize sample preparation and decomposition of reactive species. While direct spectral analysis is accurate and minimizes decomposition, sensitivity and selectivity are limitations, especially in the presence of complex sample matrices or when ppm-level sensitivity is required. This paper focuses on the techniques that are widely used in the pharmaceutical industry, namely, chromatographic separations followed by inline detection, and describes the considerations necessary to apply these techniques to reactive molecules.

Overview of Commonly Used Analytical Technologies

Direct analysis by reverse phase liquid chromatography (RP-HPLC) is the preferred separation technique in the pharmaceutical industry due to its ability to resolve complex mixtures with gradient elution, the extensive selection of available stationary phases available commercially, and its compatibility with a range of detectors including ultraviolet/visible (UV/VIS), mass spectrometry (MS), corona aerosol detector (CAD), evaporating light scattering detector (ELSD), etc. However, RP-HPLC 's utility may be limited when analyzing molecules that are reactive to water, silanols, or modifiers in the mobile phase such as acids, bases and buffers.

Some reactive molecules, such as boronate esters[8,9] are amenable to direct analysis using RP- HPLC conditions when special considerations are taken. However, in the majority of cases, accurate and sensitive analysis of reactive molecules requires alternate methodologies.

Derivatization has long been used to facilitate analysis of reactive molecules as derivatization serves to stabilize the molecule while often affording the opportunity to increase method sensitivity. Common derivatizations include alkylation, silylation, acylation, and chiral derivatizations.[10] Strategies for derivatization of a myriad of compounds have been developed for both HPLC and GC analysis.[11,12] While derivatization is a valuable technique, the analytical chemist may wish to avoid it to minimize sample preparation time and to address concerns over incomplete conversions or side reactions.

Normal phase chromatography (NP-HPLC) and supercritical fluid chromatography (SFC) have also been used to analyze molecules which are not stable in the aqueous phase required for reverse phase chromatography. In some cases, mobile phase modifiers such as acids and bases may also be eliminated in NP-HPLC and SFC, thereby addressing another potential route of degradation. Normal phase liquid chromatography is not without its drawbacks, however; sensitivity of late-eluting peaks may be inadequate due to band-broadening[13] as NP-HPLC is not amenable to gradient elution. Further, isocratic elution lacks the resolving power required for complex mixtures. SFC addresses this limitation, thereby allowing the analytical chemist to use normal phase solvents with gradient elution. Although SFC is once again gaining rapid adoption, this separation platform is not as widely available as HPLC, and therefore RP-HPLC may still be preferred to SFC. It should be noted that both NP-HPLC and SFC are limited to molecules that can be dissolved in normal phase solvents.

Finally, gas chromatography (GC) allows for both direct analysis and the analysis of derivatized species. Multiple GC detectors, including MS, thermal conductivity detector (TCD), and flame ionization detection (FID) are available, with the latter two's response reflective of concentration across analytes. While water can be eliminated from the sample prep, thereby enabling analysis of water-sensitive compounds, GC remains limited to volatile and thermally stable molecules such as sulfonate esters[14] and allyl chlorides.[15]

No single methodology is appropriate for characterization of all species of reactive molecules, but by considering the properties of the molecule and their compatibility with a wide range of analytical techniques, the modern analytical chemist can develop accurate and sensitive methods for all but the most reactive species.

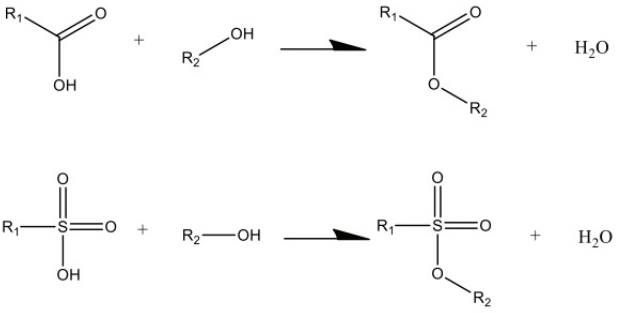
Strategy for Reactive Molecules Analysis

This paper presents several case studies where accurate and sensitive analytical methods have been developed to analyze reactive molecules. Table 1 outlines various classes of reactive molecules used in the pharmaceutical industry, their synthetic utility, mutagenic potential, and analytical strategies for their characterization.

Analysis of Acids

Acids, such as carboxylic and sulfonic acids, are widely used in the synthesis of pharmaceutical compounds. They may be a part of the synthetic scheme itself, introduced to form a salt, used as a catalyst, or charged directly to the reaction mixture to control the pH. Additionally, acids may form as a byproduct, such as during an elimination reaction.

Typically, acids are analyzed using traditional HPLC methods with little concern for their reactivity. However, use of protic solvents (methanol, ethanol, etc.) should be avoided as a sample diluent or during analysis due to potential esterification reaction with the analyte unless derivatization of the acid with the alcohol is a desired outcome.



If protic solvents are necessary to achieve an adequate separation, the short term stability of the acid in the mobile phase should be confirmed off-line, taking into consideration HPLC parameters that could enhance the esterification reaction such as elevated column temperatures

Certain sub-classes of acids represent significant analytical challenges during analytical characterization due to reactivity which necessitates the analysis of both the acid and its potential reaction products. Two of these classes are discussed in detail in the sections below:

Sulfonic Acids

Synthetic Utility

Sulfonic acids are widely used in the pharmaceutical industry to form salts of basic compounds, which modulates

Table 1. Synthesis Utility, Mutagenic Potential, and Analytical Strategy for Various Reactive molecules.

Reactive Molecule Class	Synthetic Utility	Mutagenic Potential	Analytical Strategy
Acids	Salt formation, pH control, accessing carboxamides, peptide synthesis		Avoid alcohols
Sulfonate salts/esters	Leaving groups in SN1, SN2, E1 and E2 reactions, salt formation	Alkylating agents	GC-MS
Acyl (Acid) Chlorides	Formation of carboxylic acid derivatives, Friedel-Crafts acylations	Alkylating agents	Derivatization -> RP-HPLC or NP-HPLC/SFC HS-GC
Aldehydes	Reduction to alcohols, oxidation to carboxylic acids, nucleophilic addition reactions	Alkylating agents	Avoid water, non–nucleophilic diluents -> GC
Amines	Schotten-Baumann reaction, Hinsberg reaction, alkylation, acylation, sulfonation, conversion to amines	1° & 2° aromatic amines	Salt formation -> HPLC
Boronate esters/boronic acids	Suzuki coupling, Chan-Lam coupling, Liebeskind-Srogl coupling, conjugate addition		Non-aqueous apolar diluent; low silanol activity columns or High pH mobile phases
Hydrazines	Accessing heterocyclic compounds, reducing agents, polymerization catalyst, Wolff-Kishner reduction, Sulfonation	Adduct with DNA	Avoid water, Derivatization -> GC or HPLC

the basic compounds physical and/or physiological characteristics.[16]

Genotoxic Potential

Like other acids, sulfonic acids are susceptible to transesterification reactions in the presence of protic solvents. However, unlike the other acids, the alkyl and aryl sulfonic acids esters (sulfonate esters) have potential genotoxic activity.[17-19] For this reason, if at any stage of manufacturing, the sulfonic acid comes in contact with a protic solvent such as methanol, ethanol, or propanol, the corresponding ester must be controlled at the ppm level.[20]

Analysis of Sulfonic Acids

Routine testing of sulfonate esters may not be required if pharma-ceutical companies demonstrate that the sulfonate ester is formed below the threshold of toxicological control[18,19] but chemical reasoning arguments in the absence of analytical data may not meet the requirements of regulatory agencies. Challenges to developing methods for sulfonate esters include the low sensitivity required and the instability of these compounds in aqueous media.[17-19] Because LC-UV typically lacks the sensitivity to quantify genotoxic impurities (GTIs) at low ppm levels, most methods in the literature rely on single ion monitoring (SIM) for GCMS and LCMS,[21,22] although HPLC-DAD[23] GC-FID[14] methods have been reported. Chemical and thermal stability of the sample preparation should be assessed as transesterification occurs more quickly at elevated temperatures and under acidic conditions, while base or water shifts the equilibrium away from the ester.[20]

Derivatization of sulfonate esters has been employed to improve the stability of the esters in aqueous media and to improve the sensitivity of the methods utilized in their analysis.[24.26] One such derivatization method has shown wide applicability and has been converted into a monograph method in the Eupoean Pharmacopeia. In this method, sodium iodide is reacted with the sulfonate ester in the presence of thiosulfate to form the alkyl iodide, which is readily detected by GCMS. At Genentech, this method was employed for monitoring classic sulfonate esters (e.g. methyl and ethyl methanesulfonate) as well as less widely-used sulfonate esters: the first step of the synthetic route for one development compound utilized methanol to dissolve a starting material edisylate salt; therefore, formation of the mono- and di- methyl esters of ethanedisulfonic acid were possible. The EP method was utilized to demonstrate sub-ppm levels of these esters were present in the intermediate formed in Step 1 of the synthesis and in the final API itself.[24,27,28]

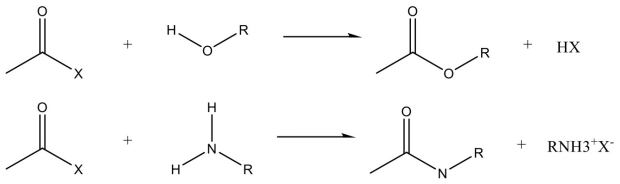
Acyl (Acid) Halides

Synthetic Utility

Acyl chlorides are widely utilized in synthesis due to their ability to form amide bonds with reactive amine groups. Additionally, the Friedel Crafts acylation allows chemists to introduce acyl substituents onto an aromatic ring.[29] Acyl chlorides also react with nucleophilic oxygen and nitrogen groups.[29]

Genotoxic Potential

Due to their high reactivity, acyl halides are alkylating agents and are thus considered genotoxic impurities. However, they are rarely of major concern in final APIs due to their high reactivity, which results in the acyl halide being purged during downstream synthetic steps and/or reaction work-ups.



Analysis of Acyl Chlorides

From an analytical perspective, acyl chlorides are extremely difficult to characterize (as starting materials or intermediates) or monitor (as potential genotoxic impurities) as they react, often violently, with water, alcohols, and phenols to produce carboxcylic acids or esters and HCl gas. In addition, they sometimes lack stability on silica, the stationary phase of most LC columns.

Normal phase chromatography or SFC may be used to successfully analyze acid chlorides, provided that alcohols and basic modifiers are not used in the mobile phase. This approach is suited to in-process control methods where the disappearance of the reactants and the appearance of the product must be monitored. It is also suitable for the release of raw materials and intermediates. See Figure 1 for a separation of an acid chloride from its corresponding acid hydrolysis product under normal phase conditions.

If appropriate selectivity cannot be obtained using acid-chloride-compatible mobile phases, or if adequate sensitivity cannot be achieved on an isocratic normal phase method, derivatization followed by reverse phase may be employed.[30,31]

Due to the reactivity of acyl chlorides, derivatization is widely reported in the literature.[32,33] Derivatization of acyl chlorides generates an analyte with adequate stability for analysis by GC for volatile species, or an aqueous-stable analyte amenable to RP-HPLC analysis. If the acyl chloride lacks a chromophore, a functional group with UV absorbance may be incorporated using 4-nitrophenol as a derivatizing agent.[34,35] When additional absorbance is not required for detection, simply dissolving the acyl chloride in an alcohol[36] quickly converts it to the corresponding ester. The derivatization product should be distinct from any product that could be created during manufacturing and/or stability conditions, eg, methanol should not be used as a derivatizing agent for a compound that contacts methanol during the manufacturing process. When monitoring acyl chlorides as GTIs, coupling derivatization with RP-HPLC and SIM-MS detection is an effective strategy to achieve ppm/ppb level sensitivity. See Figure 1.

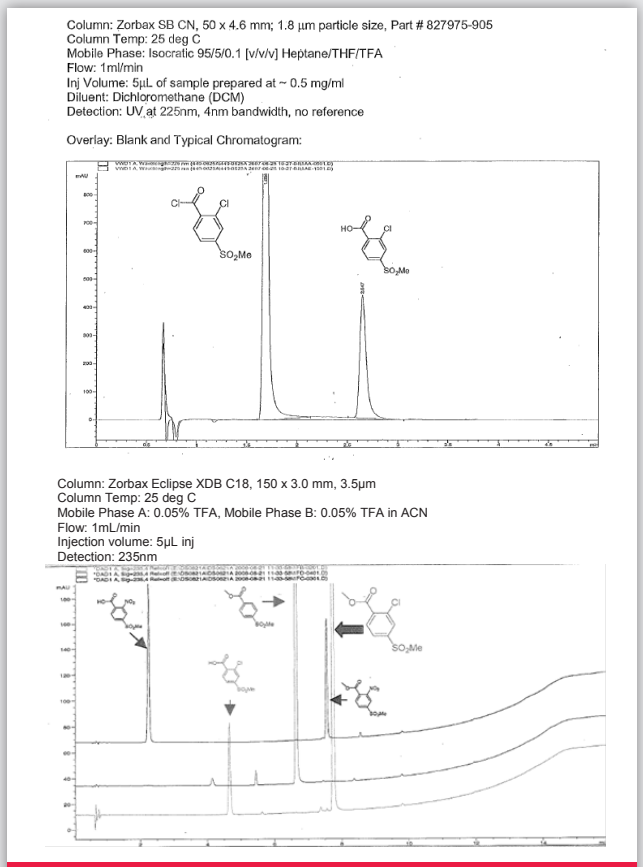
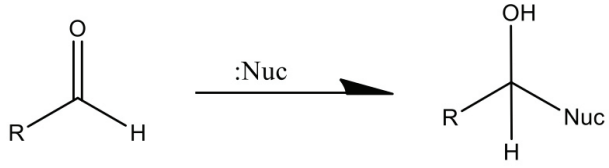


Figure 1. Analysis of Acyl halides via normal phase (top) and derivatization (bottom).

Aldehydes

Synthetic Utility

Aldehydes are another class of reactive compounds that are widely used in the synthesis of pharmaceutical APIs. Aldehydes allow chemists to access alcohols via reduction, carboxylic acids via oxidation, and serve as starting materials for nucleophilic addition reactions;[37] it's this flexibility which makes their use so prevalent.



Genotoxic Potential

Aldehydes are known alkylating agents[38-40] which can react with and therefore damage DNA.

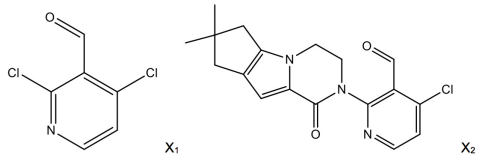
Analysis of Aldehydes

Like the previous classes of molecules discussed, the reactivity of aldehydes represents an analytical challenge for characterization. Due to the dipole of the aldehyde functional group, the carbon has a partial positive charge[41] that is subject to nucleophilic attack by molecules such as water, amines, carbon-based nucleophiles, or even other aldehyde molecules. Aldehydes have a tendency to polymerize, with unsaturated aldehydes having the greatest reactivity. In the presence of oxygen or air, aldehydes oxidize to their corresponding carboxylic acids, with rates depending on the substitution pattern. Aromatic aldehydes are more stable but do oxidize when exposed to air over long periods of time. This process is accelerated with increases in temperature. Basic conditions accelerate oxidation and polymerization, while acidic and basic conditions enhance[42] nucleophilic attacks on aldehydes.

The reactivity of the aldehyde (and thus the ability to use standard analytical techniques) must be assessed on a molecule-by-molecule basis. When an aldehyde is sterically hindered or next to electron-donating groups, characterization by RP-HPLC is a viable approach. However, for more reactive

aldehydes, alternate analytical techniques are required. Direct inject GC is an ideal methodology for low-molecular weight species because water and other nucleophiles can be avoided. However, care must be taken with diluent selection as the most reactive aldehydes may require non-polar solvents such as hexanes.

For instance, compound X1 degraded rapidly when RP-HPLC was attempted, and was ultimately characterized by GC. Compound X2, however, was successfully analyzed by RP-HPLC. In the case of structure X2, steric hindrance and keto-enol stabilization allowed for successful characterization of X2 and its impurity profile.



In addition to their chemical reactivity, aldehydes are subject to keto-enol tautomerization, and when this reaction is faster than the LC timescale, both species may elute as one broad peak. When the tautomerization is slower than the LC time scale, even fully resolved peaks may show an elevated baseline between them.[43] Heating the column may cause the peaks to coalesce.

Amines

Synthetic Utility

Amines are one of the most widely used functional groups in synthetic chemistry, enabling access to a wide array of structures. Common amine reactions include the Schotten-Baumann reaction, C-N coupling, alkylation, acylation, and sulfonation.

Genotoxic Potential

When metabolized, aromatic amines are converted to nitrenium ions that react with the nucleotides of DNA.[44-47] Therefore molecules containing this moiety are frequently flagged as GTIs and require trace-level analysis.[44,47-51]

Analysis of Amines

In general, amines are sufficiently stable for analysis by RP-HPLC. However, historically amines suffered from severe peak tailing in RP-HPLC[52,53] with the low-pH mobile phases that are desirable due to cleaner baselines afforded by low UV-absorbing additives such as phosphoric and perchloric acids. Traditionally, the lack of retention of amines due to protonation at low pH54 and the presence of peak tailing due to interaction between the protonated amine and free silanols on the silica column required RP-HPLC analysis utilizing high pH mobile phase,55 or ion-pairing reagents, e.g. octanesulfonic acid, that contribute to high baseline absorbance,[52,56,57] or chaotropic agents.[54,57,58] However, advances in RP-HPLC columns chemistries, including superior end-capping, embedded polar functional groups, and bi- and tri-dentate stationary phase binding, mean that superior peak shapes can now be obtained for amine compounds using RP-HPLC and low pH mobile phases.[53,55,59] Newer mixed mode reverse phase/cation-exchange stationary phases have been reported to improve the peak shape of basic analytes at low pH,60 and a greater number of stationary phases stable at high pH are now available for use with amine compounds that fail to give good peak shape at low pH even on the best available columns.[55,61] As an added benefit, working at a pH well above the amine's pKa may also improve repeatability of the assay.[62]

Derivatization of amines is a well-established strategy, especially for highly reactive amines and amines without chromophores. Several well-established derivatization reagents are commercially available, including acylating and silylating agents suitable for primary and secondary amines. More information on derivatization is available in literature.[63-66]

SFC separations of amines are subject to similar considerations of column technology and mobile phase pH as RP-HPLC separations. However, supercritical CO_2 has been shown to react with amine groups to form the corresponding carbamic acid, with primary amines reacting the fastest due to the absence of steric hindrance. Choosing methanol, which preferentially reacts with CO_2 to form methylcarbonic acid, as a mobile phase, will protect the amino group of the analyte.[67]

Aryl amines are relatively unstable (reactive) and are subject to oxidation/degradation when exposed to air, especially when in solution.[68] Salt formation of aryl amine often improves their stability for long-term storage as solids,[69,70] and the process chemist may conduct reactions in organic solvents in an oxygen-free environment to avoid degradation. However, exposure to air and water are largely inevitable in the analytical laboratory, making aryl amines a challenge to characterize.

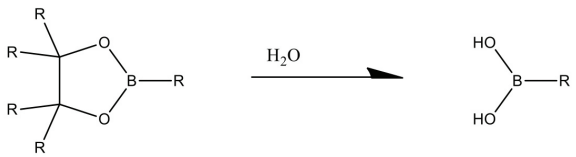
In one extreme case at Genentech, 2-amino-5-fluorobenzene-1,3-diol, a resorcinol compound, was a starting material used in GMP synthesis. It posed a significant analytical challenge for characterization: rapidly degrading, dimerizing, and even trimerizing in solution.[71] In addition to its lack of stability, adequate retention could not be achieved on a traditional C18-based column, necessitating the use of a mixed-mode column. A weak cation exchange column gave symmetrical peak shape and adequate retention.

Because the amount of degradants (a/a) increased with the age of the sample preparation, efforts were focused on stabilizing the sample preparation. Multiple diluents (THF, stabilized and unstabilized, ACN, hexane, isopropyl acetate (IPAC)), were screened, with IPAC affording the best solubility and stability. Because solubilizing in IPAC slowed, but did not eliminate degradation, derivitization agents were screened, including methylbenzyl isothocyanate, phenylethyl isothiocyanate, densyl chloride, Fmoc, Boc anhydride, and acetone. These reactions generated multiple side-products and incomplete conversion. Antioxidants such as n-propyl gallate and tocopherol were investigated but failed to slow the degradation. Sparging the IPAC solution with nitrogen or argon also failed to slow the degradation reactions, as did decreasing the concentration of resorcinol in the sample solution. Ultimately, a method with a four-hour solution stability of the IPAC solution was adapted; acceptable as a phase-appropriate characterization strategy. See Figure 2.

Boronate Esters

Synthetic Utility

Boronate acids/esters are widely used in synthetic chemistry in Suzuki coupling, Chan-Lam coupling, Liebeskind-Srogl coupling, conjugate addition, Diels-Alder and C-H functionalization.[72,73] Although the boronic acid is the active species in these reactions, the more stable boronate esters are often utilized in a biphasic (organic/aqueous) reaction medium due to their stability under reaction conditions and the ease of characterizing their stoichiometry.[74] However, the synthetic advantage of rapid hydrolysis of boronate esters to the reactive boronic acids *in situ* proves to be a significant challenge for the analytical chemist attempting to analyze boronic esters by RP-HPLC.



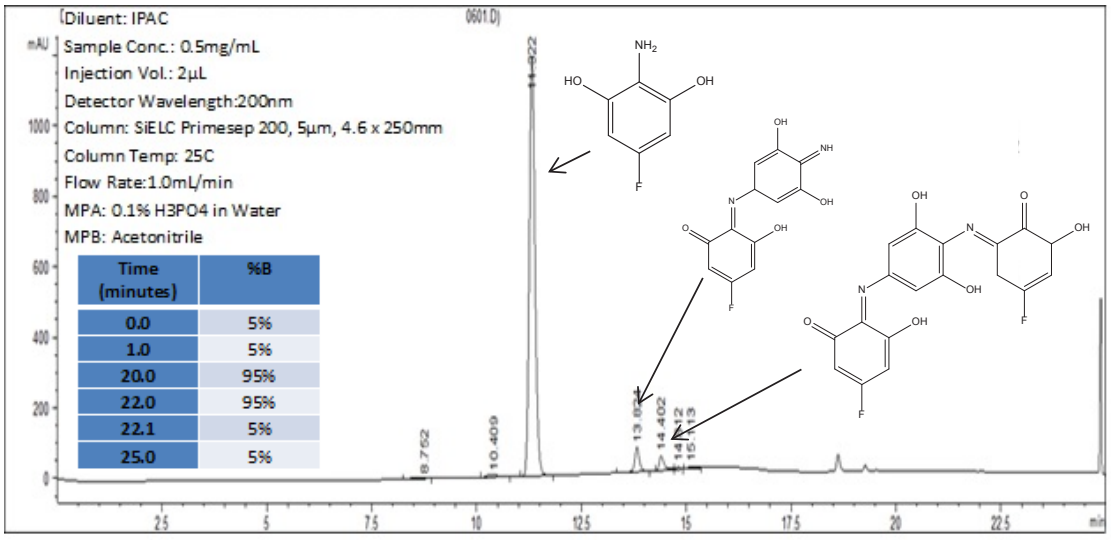


Figure 2. Analysis of 2-amino-5-fluorobenzene-1,3-diol.

Genotoxic Potential

As a class, boronate esters are not known to have genotoxic potential.

Analysis of Boronate Esters

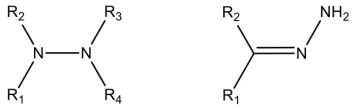
The reactivity of boronic esters with water is pH dependent,[75] which necessitates the use of non-aqueous (and typically non-protic) sample diluents below the pKa of the ester. The remainder of this section focuses on stabilizing boronic esters during the analysis itself.

While on-column hydrolysis of boronate esters[8] makes them challenging to analyze, several factors that affect the susceptibility of the boronate esters to hydrolysis have been identified. At pH's greater than the pKa of its corresponding acid, the boronate ester may be stable in aqueous conditions making RP-HPLC analysis feasible.[74,76-78] Additionally, electron-donating groups on the aromatic group of a boronate ester can slow hydrolysis by decreasing the Lewis acidity of the boron atom.8 Steric effects also strongly affect the rate of hydrolysis of these esters: greater steric hindrance of the boron atom affords greater resistance to hydrolysis.[74,79,80] Regardless of the above stabilizing factors, care should be taken to minimize on-column degradation: modulating the column temperature, the initial gradient composition, and the length of analysis can be utilized to reduce the degree of hydrolysis.[81]

For boronic esters that are relatively resistant to hydrolysis, low pH RP-HPLC separations are possible. Hydrolysis is mitigated in the absence of the silanols that commonly occur in silica-based HPLC columns. In a recent study, selection of an RP-HPLC column with low silanol activity, e.g., the XTerra MS C18, allowed the successful analysis of a variety of boronate esters.[8]

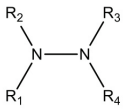
Alternate approaches are required for boronic esters which are highly susceptible to hydrolysis. The use of a high-pH mobile phase (pH 12) enabled RP-HPLC analysis of such a boronate ester.[9] A significant hurdle in this method was the retention of the corresponding boronic acid. Due to its hydrophilic nature, the acid is not retained well at the high pH necessary to stabilize the boronate ester. Polar embedded and mixed mode stationary phases, which increase retention of highly polar analytes, are not compatible with the high pH mobile phase required for this molecule. Instead, an ion-pairing reagent was added to the mobile phase to retain the acid impurity.9 In similar cases, a glucaminium-based ionic liquid[82] was used to increase retention of the boronic acid.

Hydrazines/Hydrazones



Hydrazine Hydrazone

R = H, Alkyl group



Hydrazide

R_1 = O, R_2, R_3, and R_4 = H, alkyl group

Hydrazines

Synthetic Utility

Hydrazines represent a class of reactive compounds which are widely used in pharmaceutical synthesis and for which analytical characterization is problematic. This class of compounds is used in the formation of heterocyclic compounds requiring nitrogen-nitrogen linkages.[83-87] They may also be utilized as reducing agents, in Wolff-Kishner reductions,[88] and sulfonation reactions.

Genotoxic Potential

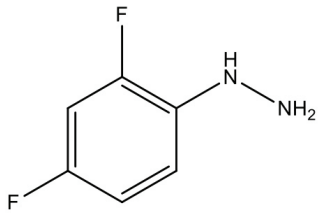
Hydrazines are frequently flagged as GTIs. They test positive in the Ames bioassay and are considered potentially carcinogenic in humans, though animal studies showed a significant increase in tumors.[89-91]

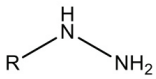
Analysis of Hydrazines

Challenges to the analytical chemists in the analysis of hydrazines include lack of chromophores, lack of retention in reverse phase and gas chromatography, low molecular weight, thermal instability, and the reactivity of the molecules. These factors contribute to poor responses by UV, CAD, FID, and MS. In addition to these challenges, their genotoxic potential requires analysis to

the low ppm level. Finally, the analytical chemist should be aware of the potential explosive hazards of this high energy molecule.[90,92]

The poor response of hydrazine to UV and MS detectors and its lack of retention can be mitigated by derivatization. Hydrazine derivatization methods provide ppm and even ppb levels of detection by improving retention and adding chromophores or MS-ionizable groups,[93-96] though hydradzines substituted with larger R groups such as isopropyl or dimethyl may make derivatization hard due to sterics. Many derivatization methods have been developed for use in environmental testing,[97-101] and may be applicable to pharmaceutical analysis of substituted hydrazines with modifications to the sample preparation procedure. Analysis of underivatized hydrazine has also been reported in the using alternate retention mechanisms such as ion, ion-exclusion, ion-pair, and HILIC and non-traditional detectors such as CLND and amperometric, conductometric, and potentiometric detectors.[102-104]





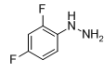
R= h, alkyl

In one case at Genentech, a phenyl hydrazine was used in the synthesis of an early stage project. The R group was non-polar, and adequate retention by HPLC was obtained using a polar-embedded column. The sub-ppm levels desired could not be achieved with UV detection or even MS detection of the parent ion. However, the sensitivity of the method was improved over 10-fold by utilizing MS/MS and monitoring the daughter ion. This technique demonstrated the residual hydrazine to be at adequately low levels in the intermediate formed from the reaction with the hydrazine and was shown to be absent in the final API. Figure 3.

Final LC/MS/MS Method for (2,4-difluorophenyl)hydrazine (DFPH

- Column: Hypersil Gold Aq, 150 x 4.6 mm, 3 um
- MPA: 98% water / 2% ACN / 0.1% formic acid
- MPB: 98% ACN / 2% water / 0.1% formic acid
- Flow rate: 1 mL/min
- Injection volume: 25 µL
- MS detection using Thermo LTQ ion trap
  - ESI, positive ion mode
  - Monitoring 145.0 / 125.0 m/z
- Column temperature: 25°C
- Gradient:

Time (min)	% MPB
0	5
7	16
8	100
12	100
13	5
18	5



Molecular Weight: 144.12

Figure 3. MS/MS of daughter ion for sub-ppm detection of (2,4-difluorophenyl)hydrazine.

Conclusion

In this review, we have provided a toolkit of analytical techniques and approaches to enable the analysis of reactive molecules used in the synthesis of pharmaceutical products. We have demonstrated that the while derivatization remains a valuable tool for analyzing the most reactive intermediates and starting materials, the analytical chemist can often use the industry-preferred separation and detection by HPLC. Eliminating or reducing components of the mobile phase known to react with the analyte of interest is frequently successful, as is selecting columns with less reactive stationary phases and decreasing overall analysis time. GC remains an important tool, and SFC continues to gain adoption across the industry. By leveraging this range of analytical techniques, the analytical chemist can deliver a high quality, reproducible test methods capable of obtaining repeatable and robust analysis to ensure high quality products and patient safety.

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Mass Spectrometry in Small Molecule Drug Development

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Abstract

Drug discovery and development is a labor-intensive and time-consuming process that comes with a significant price tag. Mass spec-trometry (MS) technology has evolved to the point where it is used throughout the drug development process, and now plays a key role in advancing the production of pharmaceuticals. In particular, when MS is coupled with a chromatographic separation technology, it becomes a powerful analytical tool, which adds an orthogonal detection function for sample analysis, and provides information-rich assessment of pharmaceutical compounds. This review describes the strategies and current approaches for MS and hyphenated MS in supporting of small molecule drug development. It also highlights the latest developed instrumentation and software that has great potential to expand the utility of MS for pharmaceutical development.

Introduction

In spite of the great progress made in research and development to combat severe diseases such as cancer, rheumatoid arthritis, high blood pressure, and aging-associated diseases, the drug development process itself has become increasingly complex and expensive. On average, it takes approximately ten to twelve years and $1.4 billion to bring a new drug to market[1,2]. It is estimated that only one drug reaches market approval for every 5000 new chemical entities evaluated in a discovery program. Drug development generally includes four major stages: drug discovery, preclinical development, clinical development, and commercial manufacturing. The longest stage is typically clinical development, which encompasses the testing done in humans (i.e. Phase I to Phase III). One crucial step is the proof of concept study for efficacy, which is performed early in drug development and is a key decision point and can lead to termination of a drug discovery program of five to seven years' duration[1]. Compared to ADME/DMPK, the use of mass spectrometry (MS) in early phase drug development is not well documented. This in part can be attributed to the regulatory requirements in drug development, which limits the development and acceptance of novel methods[3]. With the recent development in both software and instrumentation, MS techniques have been well adapted and are now the preferred choice for many applications

in pharmaceutical development[4,5]. Furthermore, new technology is needed to support novel therapies and more stringent regulatory requirements, which requires highly sensitive methods providing full profiles of drug and impurities during development. MS technology has evolved to meet this need and is emerging as the tool of choice for many applications in drug development.

MS is often considered the most sensitive detector and is typically coupled with other technologies, most commonly gas chromatography (GC) and high-performance liquid chromatography (HPLC), but also with supercritical fluid chromatography (SFC), inductively coupled plasma (ICP), ion chromatography (IC), ion mobility spectrometry (IMS) and capillary electrophoresis (CE). This type of orthogonal-mass spectrometric methodology has facilitated drug development enormously, primarily due to the superior speed, sensitivity, and selectivity of such "hyphenated" techniques.

This review provides an overview of various applications of MS and hyphenated MS techniques in support of small molecule qualitative and quantitative analysis. It also describes the established workflows during small molecule drug discovery and development that utilize MS for high-throughput pharmaceutical compounds characterization, and impurity and degradant identification. In addition, some newly developed technologies in MS are discussed for their future application within pharmaceutical development.

General Applications of Mass Spectrometry in Small Molecule Drug Development

MS is an essential tool in determining the molecular mass information of interest by ionizing chemical compounds to generate charged molecules or molecule fragments. The most common forms of ionization in small molecule research are electron ionization (EI), atmospheric pressure chemical ionization (APCI), and electrospray ionization (ESI). EI and APCI have a limited upper mass ranges (< m/z of 1,000), while ESI, and matrix-assisted laser desorption ionization (MALDI) have a high practical mass range. As illustrated in Figure 1, ESI is better suited to higher-molecular-weight and polar compounds, while APCI is best suited for low- to medium-polarity compounds. EI is typically used in GC/MS for small, volatile molecules.

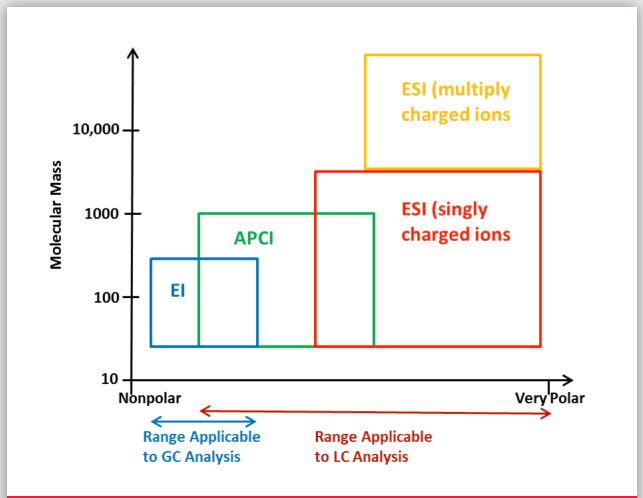

Figure 1. Common ionization techniques and application areas

Ambient ionization technologies, a terminology coined by professor R. Graham Cooks at Purdue University[6], refers to a class of sampling ionization techniques for direct ionization of chemicals from samples in their raw or unprocessed "ambient" state using either spray, heat, plasma, high electric field, or laser impact. The potential value of ambient ionization was demonstrated with desorption electrospray ionization (DESI)[6] and direct analysis in real time (DART)[7], as well as another 30-plus ambient ionization methods developed thereafter[8,9]. All these technologies have shown that ambient MS can be used as a rapid tool to provide efficient desorption and ionization with minimal sample preparation in various areas, from pesticides identification on the surface of fruit[10], to residual illicit drugs detection on the surface of paper currency[11]. Impressive results also have been achieved for chemical reaction monitoring to elucidate reaction mechanisms by MS coupled with DART[12] and DESI[13,14] ionization. Ambient ionization is also a powerful analytical tool for the rapid identification of APIs on the surface of tablets, which is important for analysis of diverted pharmaceuticals or counterfeit products[15].

For the analysis of complex mixtures, hyphenated techniques, such as HPLC-MS and GC-MS, are used and provide a wealth of analytical information. GC-MS is commonly used to analyze volatile compounds. GC-EI-MS produces reproducible spectra across instruments and labs, and the spectra can be readily searched against commercial libraries for identification of unknown compounds. When MS is coupled with HPLC/UHPLC, it is added as an orthogonal detection

technique to UV detection to provide both mass information and quality assessment of pharmaceutical compounds.

Supercritical fluid chromatography (SFC) coupled with MS has provided a valuable tool in a wide range of applications[16], including chiral separation, achiral separation, and mass-directed fraction collection in preparative SFC[17]. As the SFC technology matures, there has been an increase in SFC-MS applications for both analytical and preparative areas, in relative to traditional normal phase methods, due to the speed and reduced waste[18].

Other more specialized methodologies have been evaluated for the separation of structural isomers and chiral compounds. Dwivedi et al. has demonstrated that by coupling ion mobility spectrometry (IMS) with MS and employing a chiral modifier to the buffer gas, enantiomers can be isolated in the gas phase[19]. In another study, Rudaz et al. demonstrated that chiral separations and identification of enantiomers could be achieved by utilizing Capillary Electrophoresis Electrospray Interface for MS (CESI-MS)[20].

Ion chromatography (IC) has been extensively used as a com-plimentary separation technique to HPLC. It provides efficient separation of charged ions and polar molecules based on their affinity to an ion exchanger[21]. Recent applications include coupling to MS for inorganic ion analysis[22] to identify ions such as fluoride, chloride, nitrite, nitrate, bromide, sulphate and phosphate. Burgess et al. demonstrated that IC-MS provides sensitive detection of polar molecules, including nucleosides and nucleotides, which were typically separated by MS-incompatible ion-exchange chromatography or ion-pair reverse-phase HPLC[23].

The identification and quantitation of potential metal contamination in active pharmaceutical ingredients (APIs) is essential in drug development. Inductively coupled plasma mass spectrometry (ICP-MS) is the technique of choice for elemental determination, especially for heavy metal analysis in APIs[24]. It offers many advantages including small sample size, element specific information, rapid sample throughput, and higher sensitivity for catalyst metals such as Pd when compared to ICP optical emission spectrometry (ICP-OES). As of December 1, 2015, the United States Pharmacopeia (USP) endorses the application of ICP-MS for identifying and quantifying elemental impurities in API in chapters <232> and <233>[25,26]. The coupling of ICP-MS with HPLC solves even more complex separation problems[27], providing valuable information for unambiguous species identification.

Mass Spectrometry Analysis in Drug Discovery Chemistry

Drug discovery involves rapid testing of compound ideas and requires short cycle times from compound design to synthesis to testing, with the testing results being used for the next compound design. Typically many compounds are synthesized and tested for each discovery project until a suitable clinical candidate is selected. Analytical chemistry plays a key role in ensuring that each compound of interest (COI) has the correct structure and meets purity requirements. It is essential that analytical chemistry not be a bottleneck in the drug discovery process, so analytical labs typically employ high throughput analysis with automated data processing and reporting. Figure 2 shows a schematic diagram of a sample workflow in discovery analytical chemistry laboratory where LCMS provide essential measurement for accurate sample identification and purity assessment. A more detailed discussion can be found in the review paper by Lin et al.[28].

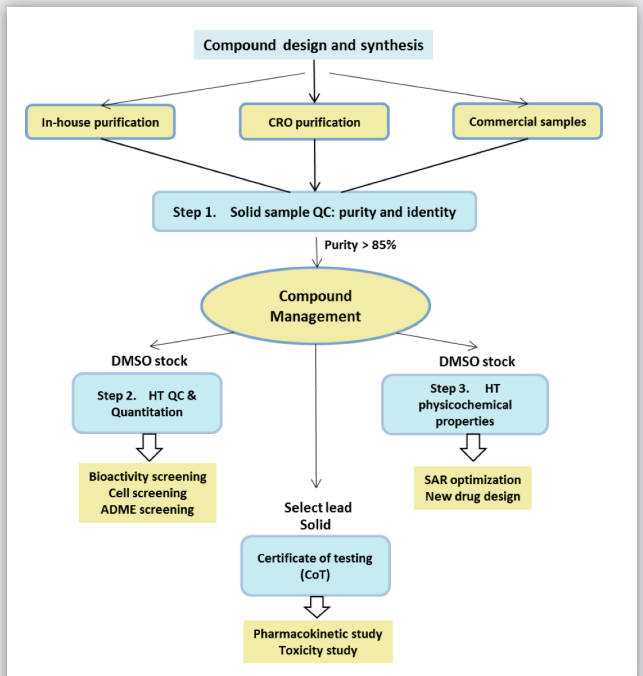

Figure 2. Workflow and processes for QC and characterization (blue boxes) in support of small molecule drug discovery in a pharmaceutical company. Reproduced with permission from Lin et al. (2015).
HT= high-throughput, CRO = Contract Research Organization, PK=pharmacokinetics, ADME = Absorption, distribution, metabolism and excretion, SAR = Structure-Activity relationship.

Large pharmaceutical companies routinely test tens of thousands of compounds that possess a wide range of properties to meet the requirements of different disease indications. A challenging area of high throughput analysis is selecting an appropriate method for each type of molecule. Samples can be small polar fragments, organic synthetic intermediates, racemic mixtures or single stereoisomers, organometallic complexes, peptides, or linkers and payloads of antibody-drug conjugates.

Table 1 summarizes the high-throughput analytical methodologies used to assess compound purity and identity. The purity profile for COIs is determined by UHPLC chromatography coupled with a diode-array detector. Structure confirmation for COIs often includes high-resolution mass spectrometry using both ESI positive and negative ion detection modes. Compound quantification from solutions, needed for quality control of compound DMSO stock solutions as well as physicochemical assays, is determined by LC-MS coupled with one or more universal detectors, such as a charged aerosol detector (CAD) or chemiluminescent nitrogen detector (CLND).

Table 1. Summary of MS methodologies for purity determination and identity confirmation. Reproduced with permission from Lin et al. (2015).

Sample	Separation Science Mode	Purity determination	Identity confirmation beside retention time matching
Small organics/ amino acids	HPLC Normal-phase Reversed-phase (C18)	UV (chromophoric) CAD, ELSD (non-volatile) GC/MS (volatile)	ESI/MS (polar) APCI/MS (neutral) GC/MS (volatile)
Mid-size organics	Reversed-phase HPLC (C18)	UV (chromophoric) CAD, ELSD (non-chromophoric)	+ESI (basic) -ESI (acidic) APCI (neutral)
Large-size organics/peptides	Reversed-phase HPLC (C8, C18)	UV CAD, ELSD	+ESI/MS (basic) -ESI/MS (acidic) HRMS
Chiral organics	Supercritical fluid chromatography (SFC) Normal phase	UV CAD	ESI/MS APCI/MS

Identification and Characterization of Impurity and Degradant for Product Development

Mass spectrometry is widely used for analysis of impurities and degradation products due to its high sensitivity and selectivity. A general MS-based strategy to analyze small molecule impurity and degradant is shown in Figure 3.

At the early stages of the drug development, rapid analysis methods that provide nominal molecular weight data are commonly used. Nominal mass information, along with the process chemist's knowledge of the synthetic scheme and associated chemistry, is usually adequate to propose structures of impurities.

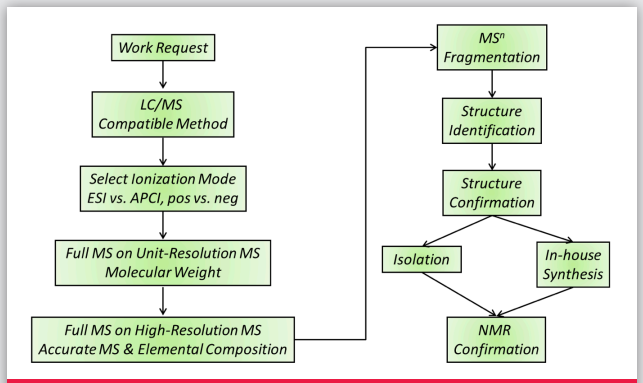

Figure 3. Strategies for identification of impurity and degradant from drug substrates and products.

As a project progresses through clinical development, the structures of unknown impurities are required and nominal mass measurements are no longer sufficient to elucidate these structures with sufficient confidence. Accurate mass is used to determine the elemental compositions of impurity structures, an essential step in elucidating the structures of unknown compounds. There are several different types of mass spectrometers capable of providing accurate masses, including magnetic sector, time-of-flight (TOF), orbital trap, and fourier transform-ion cyclotron resonance (FT-ICR) systems. In addition to advanced instrumentation, software can also help extend nominal mass data to high-resolution data by using a post-acquisition approach to calibrate mass spectral accuracy developed by Wang et al.[29].

Additional structural information can be obtained from tandem MS instruments, such as ion trap, triple-quadrupole, and Qtrap systems. The molecular ions are fragmented in space or time within the mass spectrometer, and the resulting neutral losses by MS^n processes are informative for structure elucidation of various chemical/functional groups on target molecules. This greatly facilitates the understanding of the ion fragmentation pathway for an unknown species and enables the identification of unknown compounds. Moreover, accurate mass data on fragment ions can provide additional evidence to support structural assignments.

One challenge in elucidating the structure of unknown compounds using MS is that non-volatile buffers, which are not amenable to MS ionization, are often required for isolation of the COI. In this case, the two dimension (2D)-LC-MS can be used to overcome this issue and has the added advantage of improved chromatographic resolution[30,31]. The first LC dimension utilizes the original LC isolation method and the analytes of interest are stored in loops/vials. The second dimension then uses LC-MS compatible solvents to deliver the isolated analytes from the first dimension to the MS for analysis.

To support proposed structural assignments, some straightforward chemical derivatization experiments can be performed, such as $TiCl_3$ reduction. $TiCl_3$ is typically used to reduce N-oxides degradant back into the parent molecule[32,33] and is commonly used during drug metabolites identification. It can also be used to reduce other oxidative degradants such as peroxides. Another structurally useful experiment is the hydrogen/deuterium (H/D) exchange reaction which can be used to measure the difference in MW of a compound before and after deuterium exchange. It confirms the number of solvent-exposed, exchangeable hydrogen atoms in a molecule, further confirming a proposed structural assignment.

Normally, LC-MS data alone does not provide a definitive structure assignment. NMR spectroscopy is needed to unambiguously identify unknown and novel compounds. However, NMR is relatively insensitive (~ 1,000x less than MS) and it can be time consuming and expensive, if not impossible, to obtain enough compound for complete NMR analysis. It is for this reason that advanced MS techniques are essential to provide as much confidence as possible for every structural assignment.

Quantitative Analysis by Mass Spectrometry

Coupled with HPLC or GC, mass spectrometry has become the detector of choice for superior sensitivity and selectivity in pharmaceutical compound quantification analysis. The combination of superior performance and ease of use has led to widespread adoption of LC/GC-single-quadrupole MS systems in regulated laboratories.

Triple-quadrupole MS instruments are prevalent in small molecule bioanalytical labs due to their high sensitivity. The most common method used in MS quantitation is multiple reactions monitoring (MRM), which selects a parent ion in Q1 and monitors its unique fragment ion in Q3. The latest triple-quadrupole LC-MS system can detect impurities well below the limits required by regulatory authorities for potential genotoxic impurities (PGIs). This is illustrated in Figure 4A where simultaneous analysis of four PGIs for one pharmaceutical compound was achieved by using HPLC-MS/MS in MRM mode. Cleaning verification (CV) also demands highly sensitive analytical methods. HPLC-MS/MS method is well established as a versatile tool for quantifying known compounds in the solvent rinsates or swabbing extracts from manufacturing equipment[34]. This is especially useful when dealing with cleanout testing for high potency drugs, i.e. human health criteria (HHC) category 3 and 4 compounds, where the acceptance criteria requires low ng/mL detection.

Although LC-MS/MS has long been recognized as a state-of-art, high-sensitivity tool for quantitation, HRMS is showing promise[35-37], particularly where efficiency and fit-for-purpose quality are critical. In full scan HRMS experiments for small molecule quantification, selectivity is achieved by creation of extracted ion chromatograms (EIC) of quasimolecular ions of the compound of interest, with a narrow mass-extraction window. The more narrow the setting of the mass-extraction window, the higher the selectivity. This is illustrated in Figure 4B where the chromatogram of four PGIs was acquired on a high-resolution MS instrument at full scan mode and the data were processed by extraction of the signal from compounds with a protonated mass-to-charge ratio within a 5 ppm (part-per-million)

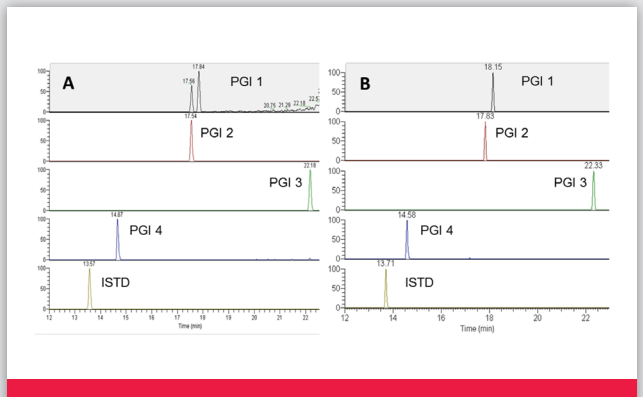

Figure 4. Chromatograms of 4 ng/mL of PGIs spiked into 4mg/mL of API. (A) The data was acquired on QqQ-MS instrument. (B) The data was acquired on high-resolution MS instrument.

Table 2. Quantitative results on QqQ-MS and HR-MS at 4 ng/mL of PGIs in the presence of 4 mg/mL API.

PGI		QqQ-MS	HR-MS
PGI 1	% Accuracy	9.0	4.4
	%RSD	1.7	3.9
PGI 2	% Accuracy	5.0	1.3
	%RSD	0.0	1.1
PGI 3	% Accuracy	14	0.0
	%RSD	15	3.8
PGI 4	% Accuracy	6.0	9.5
	%RSD	14	1.8

Based on duplicated injections.

mass accuracy window. Compared with traditional QqQ-MS, there is no significant drop in sensitivity or selectivity observed with the HRMS system, and the response is linear which enables reliable quantitation (see Table 2).

Future Perspectives in Drug Development

The recent advent of miniature/portable MS systems enables the use of MS detection beyond the analytical laboratory. A common deployment is portable GC/MS systems, where there is a need for rapid, on-site analysis of volatile and semi-volatile species important to human health, homeland security, and environmental monitoring. Miniaturized systems have also been developed to target semi- and nonvolatile species using ionization methods such as ESI and APCI. It provides a simple-to-use mass detector that can be added as an orthogonal detection technique to routine UV detection. This system has also been implemented in continuous reaction monitoring by coupling it to flow chemistry systems, allowing real-time observation of reaction intermediates at the chemists' bench[38]. Ambient MS methods, as mentioned above, when coupled with portable MS platforms[39], reduce the need for chromatographic separation and associated sample preparation.

The most common approach for identification of impurity is carried out using HPLC coupled with UV detection and mass spectrometry. However, this approach is challenging when the impurities of interest are below the UV detection limits, or low concentrations impurities are buried in the chemical noise of a mass spectrum. Advance data-mining software, predominantly used in metabolomics studies, has great potential for the discovery of chemical signatures in impurity profiling. This software is able to identify unknown impurities from noisy mass spectrograms of complex samples[40,41] Combined with powerful statistical tools, such as t-test and principle component analysis (PCA), the data analysis is relatively straightforward and manageable. The combination of this type of chemometrics software with mass spectrometry provides a powerful tool for impurity profiling during small molecule drug development.

Mass spectrometry is also showing great potential in surface analysis. MS imaging (MSI) generally refers to the use of MS for detecting the distribution of drugs and their metabolites in tissue slices[42]. It is also emerging as a technique that can provide insight into the molecular entities within cells, tissues and whole-body samples and lead to better understanding of the inherent complexities within biological metabolomes. In terms of drug development, a recent paper by Earnshaw et al. demonstrated the use of MALDI to directly image tablets[43] and the potential of this method to be used to assess the homogeneity of API in tablets during formulation development. DESI also has promise for analyzing drug tablet surfaces and has an advantage over MALDI in that no additional sample preparation is required, which could significantly eliminate potential low molecular weight MALDI matrix mass interference.

Summary

This review highlights the advantages of utilizing MS for performing qualitative and quantitative analysis of small molecules. The combination of high sensitivity, selectivity, and information-rich technology has led to MS becoming an essential tool for the analytical chemists in all stages of pharmaceutical drug discovery and development. As MS technology continues to advance and evolve, MS systems will see even wider applicability in the pharmaceutical industry.

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Are HPLC-UV Methods Fit for Purpose as True Arbiters of Quality for APIs?

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Glossary	
AIQ	Analytical Instrument Qualification
API	Active Pharmaceutical Ingredient
CpK	Process Capability
DMF	Drug Master File
HPLC	High Performance Liquid Chromatography
ICH	International Conference on Harmonisation
OOS	Out of Specification
Ph. Eur.	European Pharmacopoeia
QC	Quality Control
RSD	Residual Standard Deviation

Introduction

An evaluation of an analytical method's specificity should be performed as part of the validation process in accordance with ICH Q2 [1] and the approach used is dependent on the intended objective of the analytical procedure. In reality, certain methods may either be not specific (or not specific enough) for their intended objectives. In these cases, orthogonal approaches using two or more complementary analytical methodologies would be necessary to achieve the appropriate discriminatory power. For example, titrimetric and UV potency assays for API are non-specific and cannot detect the presence of related substances, e.g., process impurities or degradants, but have better precision (ca. 0.1-0.5% RSD) than the corresponding specific HPLC assay methods (>0.5% RSD) and therefore can trend data more effectively [2].

Are HPLC Methods Fit for Purpose?

Hofer et al. [3] modeled the ability of an HPLC assay to rapidly identify significant changes (≥0.5%) in the true mean of an API assay determination. They modeled the potential scenario where for the first 50 batches of a new API, the true mean potency was 99.5% and the standard deviation of the HPLC assay was 0.5%; thereafter the true mean potency dropped to 99.0%, with the same standard deviation. The modeling demonstrated that it is virtually impossible to detect the 0.5% change by trending the HPLC assay data and, more worryingly, if the assessor does indeed believe that a significant change in the process has occurred, it is very difficult to assess when this change took place. Tellingly the lack of this important information will hinder any future investigations into the root cause of that change. The authors advocated the use of a mass balance approach (100% - % total impurities), where the total impurity levels include related substances, solvents, water, non-volatile residues, residual metals, etc. They re-modeled the above simulation using a mass balance approach and confirmed unequivocally that it was relatively simple to detect both the change and, equally importantly, when this change occurred. An additional advantage of this approach is an understanding of changes that occur in the HPLC assay when reference standards are changed or there is a re-designation of the purity value of the existing reference standard. Finally, the authors identified those areas where the existing HPLC assay would still be required: (i) when utilizing API sourced from third-party suppliers, where detailed knowledge of synthesis and related substances may not be fully divulged (for example in a closed DMF) and therefore insufficient data are available to calculate the mass balance assay; (ii) where there is poor mass balance, i.e., where degradation to multiple compounds is seen; (iii) during the early development and scale-up activities, where there may be inadequate knowledge of the impurity fate profile; (iv) when monitoring a process that is insufficiently controlled, where degradation chemistry is not fully understood, where there

is the possibility that new impurities may be generated, or where there are concerns of contamination or adulteration; and (v) where there are legally enforceable requirements arising from a pre-defined public standard, for example, to support a pharmacopoeial compendial method.

Intermediate precision is the most appropriate validation parameter for evaluation of process capability (CpK) and should be assessed when proposing any specification limits, or when assessing the capability of the method when the specifications are constrained, i.e., 98.0-102.0% for APIs. The variability associated with the analytical methodology is frequently greater than the variability associated with the manufacturing process, particularly for API manufacture. Tsang [4] showed that for any proposed assay specification operating at 3σ, i.e., process mean ±3σ, a specification of ±2% (4% range) is equivalent to a total variability of 0.67%; thus the method variability needs to be at least half this value, i.e., 0.34%. Methods showing high process capability (often termed 6σ) are those where the total method deviation is ≤ one-twelfth of the total allowable spread or tolerance [2]. From the perspective of standard API specifications (98.0-102.0%), titrimetric methods have process capability of >6σ, whereas most HPLC methods have process capability of only about 3σ.

As a general rule of thumb the standard deviation (σ) of the analytical method should be less than or equal to one-sixth of the proposed specification range, i.e., 6σ capability. Ermer [5] assessed the maximum permitted standard deviation (σ) for an API or drug product assay method and demonstrated the dependence that this has on both the proposed specification range, process capability, and the number of repetitions of the assay (see Table 1).

Thus, for example, for an API assay method using duplicate repetitions to generate a mean potency value, where there is a lower specification limit of 98.0% and with a limit of 0.5% for total impurities (that is, a lower basic specification limit of 99.5%), the analytical method standard deviation should be 0.17% (or less). Even doubling the assay replicates still necessitates an analytical standard deviation of 0.64% (or less). Dejaegher et al. [2] indicated that one way of decreasing method variability was to increase the sample/standard weights fivefold (from ca. 32mg to >160mg); this then aligns the sample sizes to those typically seen for titrimetric methods where the precision is significantly better (ca. 0.1-0.5% RSD). However, Skrdla et al. [6] were skeptical of this approach, indicating that analytical balances in their organization were typically calibrated to a precision of 10.00 ± 0.03mg, i.e., an error of only ±0.1%.

Table 1. Largest permitted standard deviations (σ) for an assay method (showing the dependence on the proposed specification range and the number of repetitions of the assay; adapted from Ermer, 2001 [5])

Product type	Drug substance (% label claim)	Drug product (% label claim)	Drug product (% label claim)
Specification range	98.0-102.0	95.0-105.0	95.0-105.0
Basic‡ lower specification limit	99.5*	97.5**	99.0**
Number of assay repetitions	Acceptance limit for method's intermediate precision standard deviation		
2	0.17	0.28	0.45
3	0.45	0.74	1.19
4	0.64	1.06	1.70
6	0.86	1.44	2.30

* is based on sum of impurities, ** is based on an estimate, ‡ is the basic limit and covers only the variability of the manufacturing process and assumes that no method variability was present.

Therefore, the method validation data can impact on the analytical procedure, for example, the number of replicate determinations, size of sample/standards, or the calibration mode required [5].

Building on this initial work [5], Ermer et al. [7] used a total of 2915 assays (utilizing 44 different APIs, manufactured by several different large pharmaceutical companies and using 156 different stability studies) to establish a typical HPLC assay precision assessment. The cumulative API intermediate precision for HPLC assays was found to be 1.1% [8]. Hofer et al. [3] reported that the mean intermediate precision values for API HPLC assays were between 0.6 and 1.1%, with ranges of between 0.2 and 1.7%. This was aligned with Görög [9], who assessed the errors attributable to a drug substance HPLC assay method as being about 1%.

In the Ph. Eur., for potency assays of API, a maximum permitted HPLC system precision is defined, which is dependent on both the upper specification limit and the number of replicate injections. Using an analytical range of 2% (100.0-98.0%, i.e., theoretical mean – lower specification limit), gives an allowable precision of 0.73 and 0.85% RSD, respectively. Similarly, the FDA and Canadian guidelines recommend system precisions of not greater than 1% RSD. Kaminski et al. [10] recently assessed analytical instrument qualification (AIQ) criteria for HPLC equipment. They indicated that the allowable tolerance

for precision of injection volume from the auto-injector was proposed to be <1.0% RSD. This is again supportive of typical errors being about 1%.

Based on this significant analytical variability, and assuming an allowable API specification ranges of ±2.0% (for specifications in the range of 98.0-102.0%) or in reality -2.0% as the content of the API cannot be greater than 100.0%, several commentators [3,5,6,7,9] have expressed significant reservations about the utility of HPLC assay methods to monitor drug substance quality (to trend changes in API purity, to trend changes in API stability, release batches whose true potency is 98.0-102.0%, or reject batches whose true potency is <98.0% or meaningfully investigate OOS results, that could be attributable to method variability, not specification failures). Skrdla et al. [6] endorsed this view stating that, "assay results are simply not stability-indicating, to the degree required for most such studies to be meaningful (i.e., following ICH guidelines for the reporting of organic impurities), due to the large assay variability associated with them." The impact of method variability on OOS results is also significantly constrained by FDA's 2006 guidance, which requires that "all individual sample replicates, as well as the average, fall within the acceptance criteria" [11]. Hofer et al. [3] modeled the probability of finding a false OOS and found that this was very dependent on the method variability and the true mean of the API batch. They also observed that there was only a 1% chance of OOS results when the standard deviation was 0.6%, with a true mean of 99.4%, but this increased markedly (9-fold) when the standard deviation increased to 1%, with the same true mean. The possibility of seeing false OOS results also increases based on the number of tests performed on the same batch, for example, as is the case with routine stability testing. They also modeled this scenario. If the true batch mean is 99.6% and the method variability is modeled as being 0.6, 0.8, or 1.0% (RSD), then the probabilities of observing "false OOS" is relatively low, i.e., 0.4, 2.4, or 6.3%, respectively. Thus, for instance, if this batch is placed on stability with 5 time points (0, 3, 6, 12, and 24 months) and independent duplicate assays are performed at each time point, i.e., 10 assays in total, then the chances of obtaining a "false OOS" increase markedly, using the same true mean and the same method variabilities (0.6, 0.8, or 1.0% RSD), to 4, 22, and 49%, respectively. Therefore, for a stable drug substance placed on stability it is still very likely that "false OOS" results "will be observed within a surprisingly small number of tests." The authors [3] commented that this will waste significant resources and may result in non-productive measures, as there is nothing wrong with the batch in question; it is a statistical artefact of the method. That is, the API process is under control, the batch is stable, and the batch continues to meet specification—but this is unfortunately not reflected by the data! This of course can be addressed by registering broader specifications that are based on process capability rather than narrower specifications based on regulatory expectations. However, it is a moot point as to whether these broader, more meaningful specification ranges would ever be accepted by regulatory reviewers.

Hofer et al. [3] indicated that the "HPLC assay is more a test of a laboratory's ability to achieve high precision than of drug substance quality." Bunnell [12] agreed, stating that although the API HPLC assay gives "potency results within specification, the exact value will not be indicative of quality." Bunnell [12] also observed that it was practically impossible to meaningfully differentiate between HPLC assays that differ by ≤1%.

Hofer et al. [3] compared the data from the classical external standard HPLC assays versus the mass balance HPLC assay approach, generated on eight API batches. They found that mean assay data were similar (99.85% versus 99.75%), but the precision of the former data (pooled σ 0.55, range 0.31-0.80) was about 6 to 8-fold higher than the corresponding mass balance HPLC assay approach (pooled σ 0.09, range 0.04-0.20). Skrdla et al. [6] proposed the complete elimination of the classical external standard HPLC percent assays from routine use within stability studies, replacing with the more precise mass balance HPLC assay approach, which provides "much better (earlier and more sensitive) detection of low-level degradation products." The authors claim that the mass balance HPLC assay approach is much better aligned with the current ICH reporting practices (<0.05%) for impurities and degradation products and that its implementation can lead to better trending and significantly less OOS reporting. They indicated that this might necessitate a different approach to the validation of the mass balance HPLC assay, i.e., the use of several orthogonal methods and/or detection approaches might be required as part of a risk mitigation strategy if the standard HPLC assay method were removed from common practice.

Finally, method variability has a deleterious effect on the predicted shelf life of the API or drug product [13]. This is because the "difference between the point estimate of shelf life and its lower confidence limit depends on the width of the confidence interval, which is positively related to the amount of error." He

indicated that for relatively wide intervals, the shelf life determination is often rendered "practically meaningless," or at best extremely conservative. Magari [13] ran several simulations relating to shelf life prediction and the intrinsic variability encountered and concluded that a 1-year shelf life prediction is only accurate to ±1 month (i.e., ±8.33%). He indicated that utilizing an analytical method that is accurate with a high degree of precision would considerably reduce the shelf life error.

In conclusion, without some relaxation of the current API specification limits (typically, 98.0-102.0%) there seems little doubt that the use of the standard HPLC assay to monitor API quality (to trend changes in API purity, to trend changes in API on stability, to release batches whose true potency is 98.0-102.0%, or to reject batches whose true potency is <98.0% or to meaningfully investigate OOS results) must be approached with severe reservations. Tsang et al. [4], based on a retrospective analysis of the assay data for four different APIs from QC laboratories, as well as R&D, indicated that the default 4% specification range, i.e., 98.0-102.0% did not allow for any meaningful variation in the registered process. In fact, the authors indicated that the assay data would dictate that a 5% specification range, i.e., 97.5-102.5% was more appropriate. They concluded that the quality of the API can be more accurately assessed when HPLC potency data are evaluated holistically, with impurity data and other supporting data. Indeed, this is the original concept of a pharmacopoeial specification (at least in Europe). The Ph. Eur. [14], in discussing specificity of assays indicates that, "For the elaboration of monographs on chemical active substances, the approach generally preferred by the Commission is to provide control of impurities (process-related impurities and degradation products) via a well-designed Tests section, with stability-indicating methods, rather than by the inclusion of an assay that is specific for the active moiety. It is therefore the full set of requirements of a monograph that is designed to ensure that the product is of suitable quality throughout its period of use."

Several authors ([3,5,6]) have proposed the complete elimination of the existing HPLC external standard assay and replacement with the more precise mass balance HPLC assay approach, which provides significantly better detection of changes in API quality.

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