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Quantifying trifluoroacetic acid as a counterion in drug discovery by ^{19}F NMR and capillary electrophoresis

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Abstract

Drug discovery compounds are often isolated as salts of trifluoroacetate from preparative high performance liquid chromatography, which are then used for biological assays in order to assess their efficacy against the biochemical target of interest. It is, therefore, imperative to determine the TFA content in order to ascertain the correct formula weight and when required, to ensure that the TFA has been completely exchanged for another counterion in order to have superior pharmacokinetic properties and to avoid potential toxicity effects. In this paper, we present capillary electrophoresis and ^{19}F nuclear magnetic resonance methods for determining the TFA content of drug discovery compounds. Furthermore, these methods have been successfully applied in a high-throughput fashion, which is a key feature for general applicability in a pharmaceutical setting.

Keywords: Trifluoroacetate; ^{19}F nuclear magentic resonance; Capillary electrophoresis; Indirect UV; Quantitation; Drug discovery

1. Introduction

Trifluoroacetic acid (TFA) is a ubiquitous modifier used in HPLC mobile phases primarily due to its low UV cutoff (210 nm at 0.1%, v/v) and effectiveness as an ion-pairing agent. TFA is, therefore, a common counterion with compounds isolated from preparative high performance liquid chromatography. These compounds may be used as-is for various concentration-dependent biological assays in order to determine the efficacy of the drug discovery compound. The amount of TFA trapped as a counterion may be predicted based on the pK_a(s) of the molecule; however, this is not always straightforward with complex structures containing multiple ionizable functional groups. Additionally, the method of compound isolation and purification plays a role in the final amount of counterion present. Given the molecular weight of TFA (114 g/mol) the association of just one molecule to a compound with a molecular weight of 500 would increase the formula weight by more than 20%. Clearly, the amount of TFA associated with a compound must be determined to avoid the introduction of a significant source of error into concentration-dependent assay results. At the latter stages of drug discovery, it is frequently desirable to replace the TFA salts with other counterions that either provide superior physicochemical properties, viz-a-viz solubility, or do not possess the toxicity of TFA. Thus, confirmation of the complete removal of TFA can be vital.

TFA may be measured by a number of techniques including headspace GC using derivatization to produce the methyl ester [1,2], ion chromatography [3,4], capillary electrophoresis (CE) [5] and ^{19}F nuclear magnetic resonance (NMR) [6]. In this paper, we introduce the use of fast and facile CE- and NMR-based methods for the determination of trifluoroacetate as a counterion for compounds covering a wide range of polarities without derivatization or extensive sample preparation. The CE method offers the ability to quickly analyse minute amounts of compound while the NMR method permits the determination of both TFA content and structural elucidation of the discovery compound from the same sample. Additionally, as NMR instru-

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mentation is ubiquitous in research laboratories, this analysis can be performed on spectrometers equipped with ^{19}F capabilities, such as quatronuclear probes.

2. Experimental

2.1. Chemicals and compounds

Potassium hydrogen phthalate was purchased from Fluka. 2-(*N*-morpholino) ethanesulfonic acid (MES), myristyltrimethylammonium bromide (TTAB), pentafluoropropionic acid (PFPA, 97%), methylsulfoxide (DMSO), pyridinium trifluoroacetate, *N*-methylanilinium trifluoroacetate and (CBZ-Hydrazido) Glycine Trifluoroacetate were purchased from Sigma Aldrich. Trifluoroacetic acid (99.9%) was purchased from J.T. Baker, and 1 M sodium hydroxide and 0.1 M hydrochloric acid were purchased from Fischer Scientific. Ultrapure water was obtained using an in-house Milli-Q Gradient A-10 system. Several compounds presented in this paper were made in-house for the purposes of advancing our research programs, and thus were analytically characterized for structural integrity and purity.

2.2. NMR materials and methods

2.2.1. NMR instrumentation

^{19}F NMR spectra were recorded on a Bruker Avance-400 spectrometer operating at 376.48 MHz for fluorine and equipped with a z-gradient quadronuclear probe (QNP: ^{1}H, ^{13}C, ^{19}F, ^{31}P) and a pneumatic unit for automatic nuclei changes. The data were processed and analysed using the XWIN-NMR software package (Bruker Biospin). All spectra were recorded in an automatic fashion using a 120-unit automatic sample changer (BACS-120) and the ICON-NMR software (Bruker Biospin), which enables high-throughput data acquisition on numerous samples.

2.2.2. ^{19}F NMR data acquisition

All ^{19}F NMR spectra were recorded at 300 K in DMSO-d_6 solvent, and chemical shifts were referenced to the TFA resonance at −77.0 ppm. All ^{19}F NMR spectra were acquired with the addition of 128 scans and a spectral width of 100 ppm, using 64 K data points and a relaxation delay of 7 s. All free induction decays were multiplied by an exponential window function using a line-broadening factor of 0.30 Hz prior to Fourier transformation.

^{19}F NMR spectra were recorded in triplicate for each of the calibration standards (reference samples), which had predefined concentrations of TFA. The integrals from these spectra were then employed to create a calibration curve, which is a plot of these integrals versus the known concentration of TFA. The integration of the ^{19}F NMR signal from the most concentrated standard (9.8 mM) was arbitrarily given a value of 1, and the resonance integration of the other calibration standards were scaled relative to the 9.8 mM standard using the *lastscal* option of the XWIN-NMR software (Bruker Biospin). For each calibration standard, two independent sets of spectra and integrals were acquired in triplicate. For samples, in which the TFA content was to be determined, spectra and integrals were also acquired in triplicate.

2.2.3. Preparation of calibration standards and samples for ^{19}F NMR

For the calibration standards, a stock solution of trifluoroacetic acid was first prepared by accurately massing a few drops of trifluoroacetic acid into a flask containing a pre-massed volume (∼800 μL) of DMSO-d_6 solvent (TFA content > 400 mM). Multiple standard samples of known TFA concentration were then made by diluting the stock solution to 19 mM (129.8 μL of the stock diluted to 3 mL with DMSO-d_6) followed by further dilutions to produce calibration standards having 9.8, 4.8, 2.4, 1.2, 0.97, 0.73 and 0.48 mM concentrations, each in 600 μL of DMSO-d_6 solvent. These calibration standards were prepared in duplicate from two unique TFA stock solutions. Each compound, for which the TFA concentration was to be determined, was prepared in duplicate by accurately massing approximately 1 mg of material and then adding 600 μL DMSO-d_6 solvent.

2.3. Capillary electrophoresis materials and methods

2.3.1. CE instrumentation

The CE apparatus used was a P/ACE MDQ equipped with UV detector at 254 nm acquiring at 4 Hz and a PC running 32 Karat Version 5.0 software for system control, data collection and analysis (Beckman Coulter, Fullerton, CA, USA). All capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA) and had 75 and 363 μm internal and outer diameters, respectively.

2.3.2. CE data acquisition

The CE-based methods presented herein were adapted from a method published by Altria et al., for the analysis of succinic and maleic acids using indirect-UV detection [7]. Two methods were used, the first was a quantitative method partially described elsewhere [8]. In brief, the capillary was 50 cm to the detector with a 60 cm total length. The separation electrolyte was 5 mM potassium hydrogen phthalate, 50 mM MES, 0.5 mM TTAB, pH 5.0. The capillary was conditioned for 90 min at 10 kV each day prior to use and flushed with 1 M NaOH, 0.1 M HCl, water and then air at 20 psi for 2 min each prior to storage. Prior to sample analysis, the capillary was first rinsed with 0.1 M HCl followed by separation electrolyte at 20 psi for 2 min. A short voltage equilibration of 20 kV for 1 min was applied and followed by sample injection of 0.5 psi for 16 s. A 0.5 psi for 3 s injection of separation electrolyte was then injected followed by separation at 20 kV for 10 min with a 0.5 min voltage ramp. All indicated voltages are in negative polarity.

A second method was designed for screening purposes. This method employed a capillary that was 20 cm to the detector with a 30 cm total length. The capillary was conditioned for 30 min at 30 kV with 0.5 psi each day prior to use and flushed with 1 M NaOH, 0.1 M HCl, water and then air at 20 psi for 0.5 min prior to storage. Prior to the analysis of each sample, the capillary was rinsed first with 0.1 M HCl followed by separation electrolyte

at 20 psi for 0.5 min. A short voltage equilibration of 20 kV for 1 min was then applied and followed by sample injection of 0.5 psi for 5 s. A 0.5 psi for 3 s injection of separation electrolyte was then injected followed by separation at 15 kV for 2.5 min with a 0.5 min voltage ramp.

2.3.3. Preparation of standards and samples for CE

A stock solution of trifluoroacetic acid was prepared by massing a few drops of trifluoroacetic acid into a flask containing a pre-massed volume (2–4 mL) of the internal standard solution which consisted of either 138 or 276 ppm PFPA (for the quantitative and screening methods, respectively) in DMSO:H_2O (95:5, v/v). To prepare multiple calibration standards, the stock was first diluted to 2000 ppm and then further dilutions were used to produce the calibration standards from 12 to 800 ppm. To prepare a single calibration standard for the screening method, the stock was diluted to 400 ppm and injected. Each sample to be analysed was prepared in duplicate using 100–250 μg of material diluted into 300–600 μL. All dilutions were derived from the internal standard solution.

3. Results and discussion

3.1. ^{19}F NMR

3.1.1. ^{19}F NMR method for high-throughput determination of TFA counterion

NMR spectroscopy has historically been an important analytical method applied by chemists for primary structure verification or elucidation of synthetic compounds. Each compound has a unique NMR spectrum in which resonances of individual atoms are resolved, thus providing its fingerprint. In addition to compound identification, NMR spectroscopy has also been valuable for determining the relative concentration [11,12] of accompanying compounds (occasionally referred to as impurities) present in the sample tube via visual or resonance integration. In this work, we extend this application to monitor and quantify the relative concentration of the accompanying TFA counterion using ^{19}F NMR spectroscopy. Given that the TFA counterion content of numerous compounds must be analysed within a reasonable time in a drug discovery environment, we developed the "user friendly" and high-throughput strategy described herein.

To illustrate the capability of ^{19}F NMR for determining the relative TFA counterion concentration, an ^{19}F NMR spectrum was acquired on a compound that contained a single CF_3 group in its primary structure and a single associated TFA counterion. The ^{19}F NMR spectrum in Fig. 1 clearly shows the expected singlet resonance at −76.48 ppm for the CF_3 substituent as well as a singlet for the TFA counterion at −77 ppm. The additional salient feature in this spectrum is that both resonances have the same relative intensity and integration, confirming that there is indeed one TFA counterion per molecule of compound 1. Note also that compound 1 could not be analysed for its TFA content by neutron activation since the technique measures total fluorine content and not simply that of the TFA.

Given that many compounds do not contain fluorine atoms, the concentration of TFA can alternatively be determined by simply comparing the integration of the ^{19}F resonance of the compound of interest with a calibration curve that was previously constructed from samples having known TFA concentrations. Data for the calibration curves needs to be acquired only once, however, this exercise was run twice to ensure accuracy. The calibration curve was obtained using spiked samples having a range of TFA concentrations, and the resultant plot of the concentration versus integration resulted in a linear relation described by a correlation coefficient of $r^2 = 0.996$ using linear regression ($Y = 9.57(X) - 0.193$).

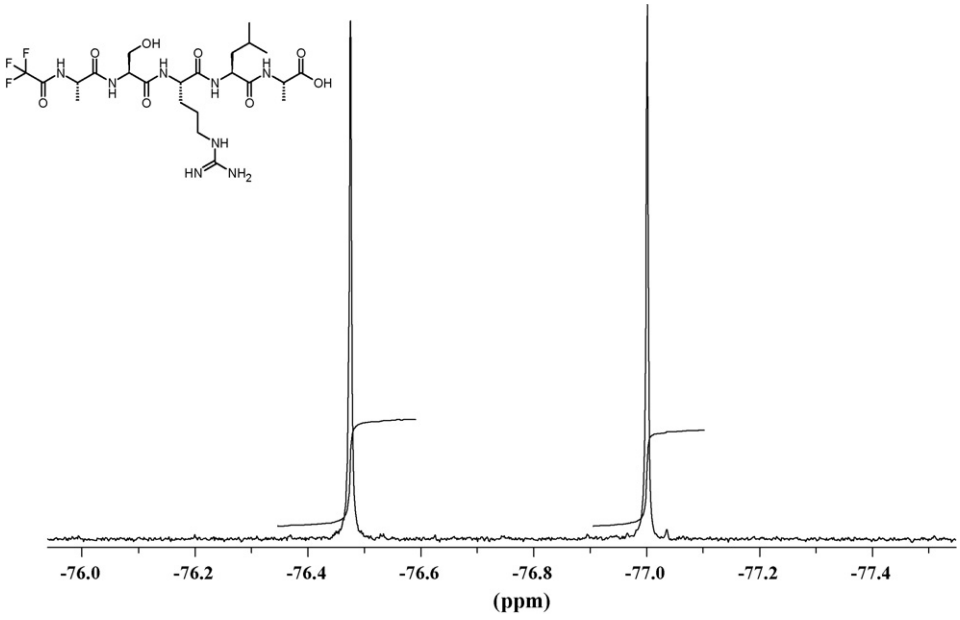


Fig. 1. ^{19}F NMR spectrum of compound 1 (also refer to Table 1).

Table 1
Intra- and inter-day reproducibility for a 2.4 mM TFA standard by ^{19}F NMR

n	Signal integral	
	Intra-day	Inter-day
1	0.255	0.253
2	0.252	0.249
3	0.252	0.250
4	0.253	0.251
5	0.25	0.258
Mean	0.253	0.252
% R.S.D.	0.568	1.35

A high-throughput strategy was then developed which also met other central criteria such as simplicity and ease of use by medicinal chemists. To enable high-throughput, our Bruker NMR spectrometer is equipped with an automatic sample changer (BACS-120) and a QNP probe with a pneumatic unit to enable easy switching between ^{1}H, ^{13}C, ^{19}F and ^{31}P nuclei. This set-up has successfully allowed medicinal chemists to acquire their own NMR data for primary structure characterization, and with no additional effort, to collect the data needed for TFA counterion determination using the same sample. Chemists need only accurately mass the compound (∼1 mg) and place it in the same volume as the standard TFA samples (e.g. 600 μL). To permit further automation, one only has to enter into a spreadsheet the integral value of the ^{19}F signal along with the weight of powder used for the sample. The spreadsheet is designed to automatically calculate the TFA concentration of the sample and the ratio of TFA to compound present. For this, the spreadsheet employs the linear regression equation mentioned above. Once this strategy was implemented, it was successfully employed to determine the concentration of TFA counterion for hundreds of compounds. For purposes of monitoring the reproducibility of the ^{19}F NMR data, intra- and inter-day reproducibility experiments are reported in Table 1. The intra-day reproducibility was measured using replicate recordings of a single 2.4 mM TFA standard and the relative standard deviation (R.S.D.) was found to be 0.6% ($n = 5$). For the inter-day reproducibility, a 2.4 mM TFA standard was prepared and analysed on different days and the R.S.D. value was 1.3% ($n = 5$).

3.2. Capillary electrophoresis

3.2.1. CE quantitation

Quantitative analyses by CE often require the use of an internal standard in order to account for injection variances due to slight deviations in the applied pressure during a sample injection. In addition, compounds synthesized in drug discovery cover a wide range of polarities and, therefore, it was desirable to have a single solvent system for which to dissolve all samples. Given the large solubilizing power and ubiquitous use of DMSO as a generic solvent in drug discovery, a solvent system-based largely on DMSO was sought. Ultimately, a solution composed of DMSO:H_2O (95:5, v/v) containing 138 ppm PFPA as an internal standard was found to be suitable.

As a result of differing ionic strengths, slight injection variations and the high concentration of DMSO in the sample diluting solvent, the migration times of the samples and standards could differ by more than 1 min from injection to injection. Fortunately, peak assignments were straightforward as the only two peaks observed are due to TFA and the internal standard, PFPA. The larger discovery compounds have longer migration times and the two most common contaminants, chloride and acetate, migrate well before and after the TFA/PFPA peaks, respectively.

The intra- and inter-day reproducibility is reported in Table 2. The intra-day reproducibility was measured using replicate injections of a single 200 ppm TFA standard and the relative standard deviation (R.S.D.) was found to be 2.7% and 2.6% for the TFA and PFPA, respectively ($n = 5$). The TFA/PFPA ratio gave 1.3% R.S.D. For the inter-day reproducibility, a 200 ppm TFA standard was freshly prepared and measured on different days over a 3-month period. The R.S.D. values were 2.9% and 4.5% for the TFA and PFPA peak areas, respectively, with 2.7% for the TFA/PFPA peak area ratio ($n = 5$).

Using four sets of calibration data from 1.60 to 800 ppm TFA, the limit of detection (LOD) and limit of quantitation (LOQ) were determined to be 3.74 and 11.3 ppm TFA in the following manner [9]:

$$\mathrm{LOD} = 3.3\left(\frac{\mathrm{SD_{blank}}}{\mathrm{slope}}\right) \quad \text{and} \quad \mathrm{LOQ} = 10\left(\frac{\mathrm{SD_{blank}}}{\mathrm{slope}}\right)$$

where $\mathrm{SD_{blank}}$ refers to the standard deviation of the blank signal and slope refers to the slope of the calibration plot. A TFA

Table 2
Intra- and inter-day reproducibility for a 200 ppm TFA standard by CE

n	Intra-day reproducibility			Inter-day reproducibility		
	Peak areas		Peak ratio (TFA/PFPA)	Peak areas		Peak ratio (TFA/PFPA)
	TFA	PFPA		TFA	PFPA	
1	64,505	35,658	1.81	65,399	36,206	1.81
2	65,353	37,081	1.76	64,363	37,104	1.73
3	68,683	38,327	1.79	63,582	36,015	1.77
4	68,275	37,428	1.82	65,413	35,685	1.83
5	66,751	37,343	1.79	60,868	32,897	1.85
Mean	66,713	37,167	1.80	63,925	35,581	1.80
% R.S.D.	2.71	2.60	1.30	2.93	4.47	2.65

concentration of 800 ppm was found to be the maximum that could be analysed due to loss of peak shape and reproducibility at higher concentrations. Based on a series of 10 calibration sets acquired over an 8-month period, a typical regression equation was $Y = 0.0093(X) - 0.0040$ with R^2 values of 0.999 between 12 and 800 ppm. A significant advantage of using the DMSO-based internal standard solution is that it is possible to prepare the sample to be analysed over a wide concentration range to ensure that the TFA concentration is above the LOQ. Thus, not only can compounds with widely differing polarities be analysed but also compounds containing only trace amounts of TFA.

3.2.2. CE quantitation via the screening method

Many analyses for TFA content in drug discovery are on preliminary compounds and as such they do not require a complete battery of analyses to assure maximum purity and characterization. In these cases, the salt content is rounded to the nearest integer of mol TFA/mol compound for the purposes of entering into a corporate compound database. In order to facilitate a fast turnaround, a shorter CE-based method was created using a 30 cm capillary and used only one calibration standard. Due to the shorter capillary, the amount of injected sample had to be reduced and as such the calibration range was re-evaluated and found to be 9.20–600 ppm with a LOD of 3.04 ppm. A single calibration standard at 400 ppm was selected for determination of the TFA content. A sample electropherogram from each of the two CE-based methods is displayed in Fig. 2. To ensure that the shorter method would provide similar results to the original, the intra- and inter-day reproducibility was evaluated for the 400 ppm TFA standard (see Table 3) and found to be 2.0% and 1.4%, respectively based on the TFA/PFPA peak ratio ($n = 5$).

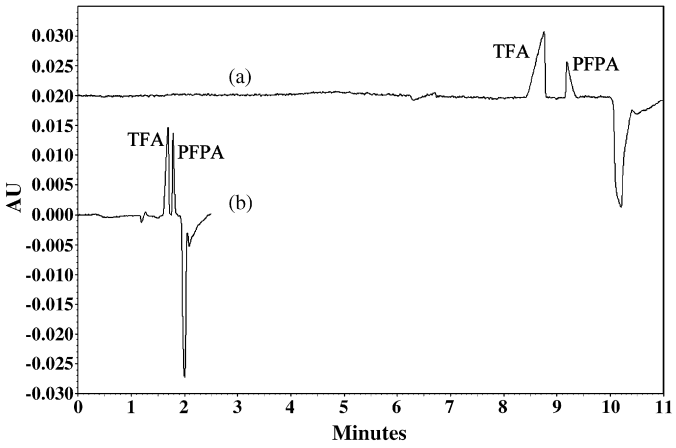


Fig. 2. Electropherograms of 400 ppm standards using the CE: (a) quantitative and (b) screening methods.

3.3. Comparison of methods for quantifying TFA content in standard samples

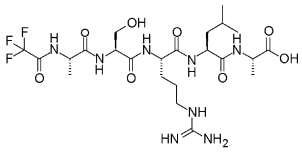
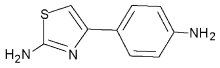
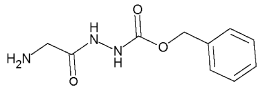
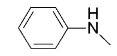
To ensure that the TFA was being determined accurately, a selection of compounds was sent for fluorine determination by neutron activation analysis whereby the amount of TFA was calculated based on the amount of fluorine determined to be present in the sample. Table 4 lists five compounds and their respective TFA content as determined by neutron activation, CE (multiple point and single point calibrations) and ^{19}F NMR. As previously mentioned, compound 1 was not suitable for neutron activation analysis as it contained covalently bonded fluorine in addition to the fluorine present in the TFA counterion. ^{19}F NMR also determines fluorine content, however differing chemical shifts permit fluorine present as TFA to be quantified independently of other covalently bonded sources (Fig. 1). The results presented in Table 4 show a general agreement between each of the methods with the average spread of the data at ±0.09 mol TFA/mol compound. Nevertheless, for compounds 2 and 5 the TFA content as determined by NMR is not in complete agreement with those determined by CE and Neutron Activation. Ruling out obvious interferences, the reason for this discrepancy is unclear, however it is not unique to ^{19}F NMR or this method as differing analytical techniques may often provide slightly differing results for the same sample. It is for this reason that in cases of complete compound characterization additional methods including HPLC and elemental analysis are used and their results are compared to one another to provide a complete picture of the characterization. As this work is intended primarily for screening the TFA content of drug discovery compounds, the results presented in Table 4 for all three techniques are reasonable.

Table 5 details four compounds from our drug discovery programs with their predicted TFA content based on their respective

Table 3
Intra- and inter-day reproducibility for a 400 ppm standard using the screening method by CE

n	Intra-day reproducibility			Inter-day reproducibility		
	Peak areas		Peak ratio (TFA/PFPA)	Peak areas		Peak ratio (TFA/PFPA)
	TFA	PFPA		TFA	PFPA	
1	66,068	33,457	1.97	66,068	33,457	1.97
2	55,095	28,967	1.90	59,109	30,047	1.97
3	54,298	27,563	1.97	58,136	29,887	1.95
4	62,803	31,312	2.01	64,612	32,144	2.01
5	57,130	29,487	1.94	66,226	32,994	2.01
Mean	59,079	30,157	1.96	62,830	31,706	1.98
% R.S.D.	8.68	7.56	2.02	6.22	5.22	1.39

Table 4
TFA counterion content of standard samples by CE (single and multiple-point methods), ^{19}F NMR and neutron activation

Compound	TFA content, w/w, % (mol/mol)			
	Neutron activation[a] ($n=2$)	CE multiple point ($n=2$)	CE single point ($n=2$)	NMR
 Compound 1	Not determined	14.8 (0.932)	14.4 (0.903)	14.5 (0.911)
 Compound 2	43.2 (1.28)	43.5 (1.29)	42.6 (1.24)	34.2 (0.878)
 Compound 3	31.5 (0.901)	31.8 (0.913)	30.6 (0.865)	32.6 (0.948)
 Compound 4	56.2 (0.891)	56.1 (0.888)	54.3 (0.826)	57.7 (0.946)
 Compound 5	47.5 (0.851)	48.5 (0.885)	47.7 (0.858)	52.4 (1.03)

[a] These compounds were analysed as described elsewhere [10].

Table 5
Selection of expected vs. actual TFA content for multiple compounds

Compound	Predicted TFA content, w/w, % (mol/mol)	Measured IC_{50} (nM)	Actual TFA content, w/w, % (mol/mol)	Corrected IC_{50} (nM)	% difference in IC_{50} values	TFA measured by
6a	13.2 (1.00)	7.70	22.4 (1.90)	6.88	−12	CE
6b	13.2 (1.00)	7.15	14.8 (1.14)	7.01	−2	
7	34.6 (8.00)	6.04	20.8 (3.97)	7.28	+17	
8	36.0 (3.00)	4876	38.0 (2.03)	5541	+12	NMR
9	12.0 (1.00)	8.10	12.5 (0.910)	8.18	+1	
10	8.29 (1.00)	1000	1.03 (0.0500)	1190	+16	
11	12.0 (1.00)	2.30	12.0 (1.00), 13.6 (1.15)	2.30, 2.26	0, −2	CE, NMR
12	21.2 (2.00)	1.30	12.4 (1.05), 12.8 (1.09)	1.45, 1.44	+10, +10	CE, NMR
13	17.1 (1.00)	14.0	0.708 (0.0345), 1.02 (0.0500)	16.8, 16.7	+17, +16	CE, NMR

structures (structures not shown for proprietary reasons), their measured inhibition constants (IC_{50}) and the results after the determination of their TFA content. The molecular weight of these compounds ranged from 550 to 1700 g/mol. At the two extremes, compound 10 was found to have 20-fold less TFA than expected while the found TFA content for compound 9 almost matched the expected value. The impact on the IC_{50} values ranged from −12% to +17%. Compounds 6a and 6b represent two different batches of the same compound prepared 4 months apart by the same chemist. The results for these two batches and also those of the other compounds highlight the importance of determining the TFA content in order to be certain of the correct formula weight and thus the correspondingly correct assay results. It is important to realize as well, that the lower the molecular weight of the compound, the larger the effect of the TFA content will have on the formula weight. Compounds 11–13 were analysed by both techniques to provide a direct comparison of results on real samples.

4. Conclusions

This report describes ^{19}F NMR and CE strategies for determining the TFA content of a variety of compounds with widely different physicochemical properties. It was demonstrated that the strategies are precise, accurate and robust, and amenable to high-throughput analyses. Furthermore, it was shown that

the accurate determination of the TFA content can significantly differ from what one would predict, which resulted in differences in corrections of inhibition constants (IC_{50}) due to differences in formula weight corrections. The importance of accurate counterion determinations was further highlighted, in that different levels of counterion were observed for different batches of the same compound. Thus, accurate TFA determinations can provide higher quality data for inhibitor activities and it is expected that the strategies presented here can provide pharmaceutical researchers with a valuable drug discovery tool.

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