JOURNAL OF combinatorial CHEMISTRY

Report

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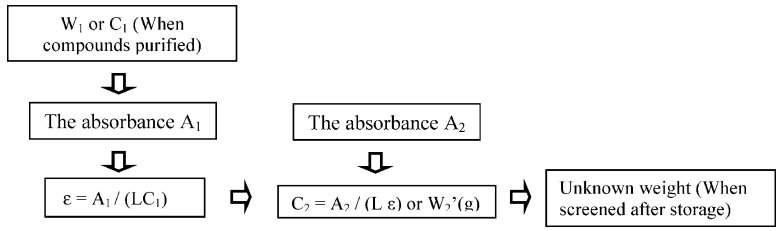
J. Comb. Chem., **2008**, 10 (2), 162-165• DOI: 10.1021/cc800007k • Publication Date (Web): 19 February 2008



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Feasibility of a Self-Calibrated LC/MS/UV Method to Determine the Absolute Amount of Compounds in Their Storage and Screening Lifecycle

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Received January 16, 2008

Screening a quality compound collection against a validated drug target is a key success factor in the lead discovery process. A compound collection, ranging from thousands to over a million individual compounds, typically contains compounds made by single-compound synthesis and those by parallel synthesis. After synthesis, purification, and the purity analysis, the quantity of compounds is gravimetrically determined before they can be registered in a collection. Compounds are usually dissolved in DMSO[1] for high-throughput screening (HTS). The remainder is stored for hit validation and future screenings over many years or even decades. The primary and secondary screenings discover hits that will be further studied by dose–response measurements to determine their IC_{50} values. The primary screening data is so crucial that a large number of compounds are denied further investigation solely based on this data. On the other hand, it is the IC_{50} value that drives the lead selection, lead optimization, and candidate advancement. However, the inconvenient truth is that the screening results have been compromised by the uncertain quality and quantity of a large number of compounds in the screening plates.

Even when compounds are pure and their weights are accurate at the time when they are deposited into a collection, knowing the final compound concentration in the HTS plates is still problematic because of at least three reasons. First, many compounds degrade with time.[1–5] Currently, there is no existing storage/process method that avoids decomposition of compounds in a collection. The instability of library compounds in a collection results in changes in compound purity and quantity that lead to the interference in assays and erroneous compound concentration. Second, to automate the distribution, dilution, and handling of a large number of compounds, a single solvent (normally DMSO) or a solvent combination such as methanol and dichloromethane is used for the automated operation. Unavoidable poor solubility of some compounds in the standard solvent causes the altered concentrations in the daughter plates. Third, biological assays are carried out in aqueous solutions. The solubility of compounds in aqueous solution is unknown and often is poor for many of them. Frequently many DMSO-soluble compounds are no longer soluble in aqueous medium. In cell-based assays, one often observes the formation of compound crystals in cell culture medium, even though the involved compounds are perfectly soluble in DMSO. The issues generate ambiguity of the final assay concentration and reduce credibility of HTS results.

Because compound decomposition is unavoidable, one should be able to determine compound purity and quantity. The standard method for quantitation of organic compounds is to use a standard calibration curve and the authentic compound as standard. This method is evidently not suitable for the inhibitory large number of compounds in any compound collection.

LC/MS with a quantitative detector is an alternative approach. Various detectors such as evaporative light scattering detector (ELSD),[6–10] charged aerosol detector (CAD),[11,12] and chemiluminescence nitrogen detector (CLND)[13–32] have been tested as a "universal detector" for the structure-independent quantitation. With the use of selective pure compound standards to make an average calibration curve, samples with the same core structure as standards can be measured quantitatively by ELSD detectors with an error of 20–30%.[8] However, when the core structure varied in cases of compound collections, the quantitation results showed significant structure dependence even using an average calibration curve made from many diverse compounds. Another evaporation-based technique, CAD, has shown better sensitivity and, in general, ELSD-like quantitation features.[11,12] The CLND detector[13–32] exhibited an equimolar response with ±10% average error for limited compounds studied.[23] Although structure dependence was observed for compounds containing adjacent nitrogen atoms,[32] overall this is the best available quantitative HPLC detector. However, the relative complex instrument operation and maintenance prevented its widespread use in analytical laboratories. Therefore an alternative, simple, and high-throughput quantitation method needs to be developed to rapidly determine the absolute quantity of compounds during the lifecycle of storage. Here we report a robust self-calibrated quantitation method based on LC/MS/UV.

The rationale for this method is simple. Compound purification has become a required step before compounds can be registered in a collection. Compounds are usually purified to a purity of 90–100% with a known weight determined gravimetrically. In the postpurification LC/MS/UV analysis, the extinction coefficient of a specific compound can be obtained from its concentration or weight and the absorbance at a given wavelength following Beer–Lambert's Law: $\epsilon = A/lc$, where A = absorbance, ϵ = molar

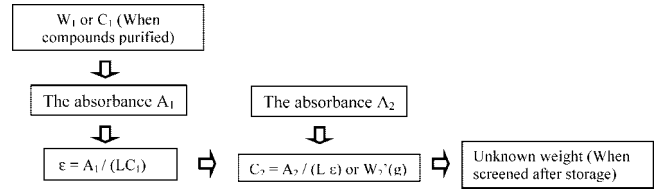

Figure 1. Flowchart of experimental procedure.

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10.1021/cc800007k CCC: $40.75
Published on Web 02/19/2008

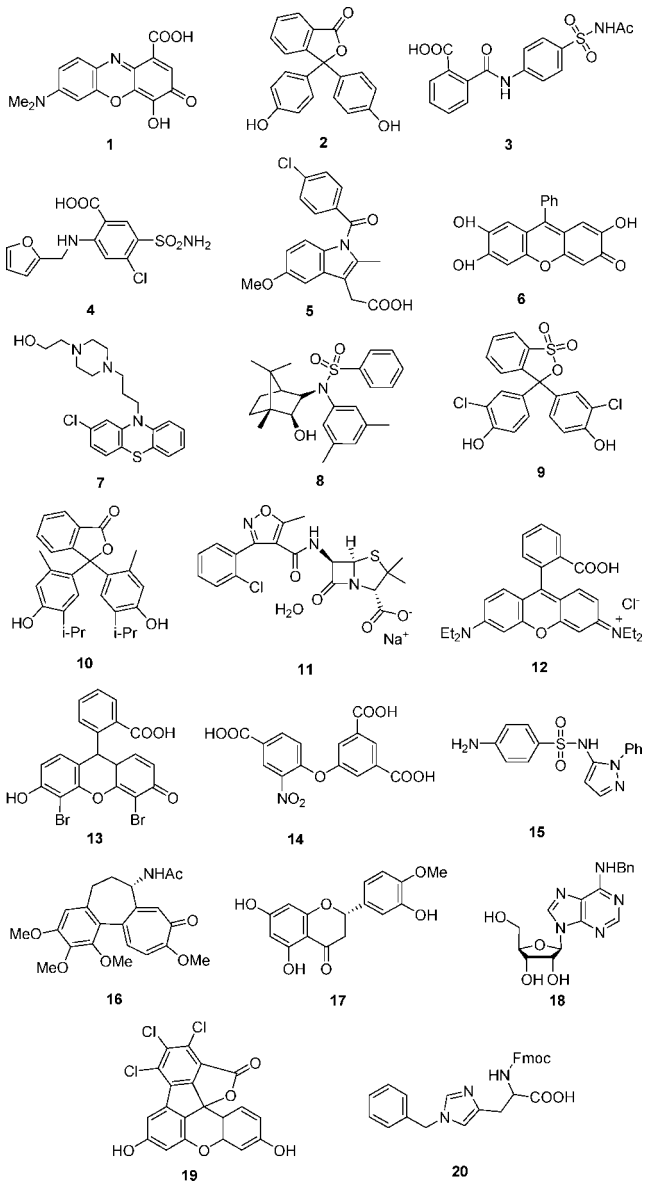


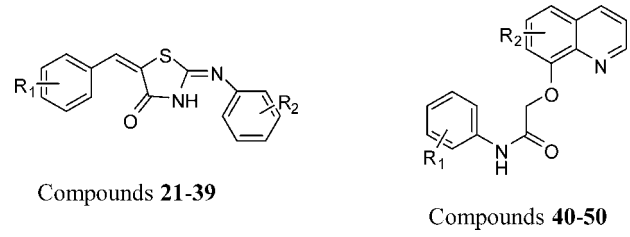
Figure 2. Chemical structures of diverse compounds.

extinction coefficient, l = path length, and c = concentration.[33] At any time during compound storage or at the time compounds need to be replated for screening, a HPLC/UV run can quickly obtain the absorbance at the same wavelength and the concentration or the absolute quantity of the compound can be derived using the previously determined extinction coefficient for this compound. Our experimental design to simulate this process is depicted in Figure 1.

We used compounds **23**, **37**, and **43**, which were selected from two libraries (structures see Supporting Information) to determine the linear range of the measurements and the reproducibility of the LC/UV method. The linear range was found to be between 4.0×10^{-5} and 4.0×10^{-3} M, and the relative standard deviation (RSD) of the nine repeated analysis of the same compound was 4.5%.

We next tested the self-calibrated quantitation method using 20 diverse compounds (**1**–**20**, Figure 2) with diverse structures and 30 purified library compounds containing two different core structures that were distinct from the diverse set (Figure 3). Similarity calculations showed that compounds **1**–**20** were highly diverse based on their Tanimoto coefficients (Table 1). We first determined their absorbance in LC/MS/UV_{214} measurements and calculated the extinction coefficients for all 50 compounds from solutions with accurate concentration. Another set of the same 50 compounds was measured as a single-blind experiment. Without knowing the compound weight and concentration (prepared by a different person), the experimenter determined the absorbance using LC/MS/UV_{214} and calculated the concentration and weight using the extinction coefficients obtained earlier. Experimental results were compared with their actual weight in Tables 2 and 3. The relative standard deviation on the estimated weight of 74% of compounds fell below 20%, and 24% of them were between 20 and 30%. The relative deviation is $10.6 \pm 8.0\%$ for the diverse set and $13.5 \pm 11.0\%$ for the library set. Because the compound quantitation was self-calibrated, library compounds with the same core structure did not offer any better accuracy. This is different from ELSD- or CLND-based universal quantitation protocols. Therefore, this method is particularly suitable for diverse compound collections.

As a demonstration of an application of this method, we carried out a forced decomposition experiment on 18 compounds (Table 4) and determined the compound loss using the predetermined extinction coefficient. When the samples were heated to 200 °C, we found that compounds were stable with little decomposition. When compounds were treated with sulfuric acid (98% v/v), decomposition was detected. The compound loss was determined by the self-



Compounds **21-39** Compounds **40-50**

Figure 3. Chemical structures of library compounds.

Table 1. Similarity Analysis[a] of the Diverse Compound Set in This Study

	comparison reference compound									
compd	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	1.00	0.40	0.33	0.23	0.33	0.45	0.28	0.15	0.55	0.24
2	0.40	1.00	0.27	0.28	0.54	0.47	0.48	0.12	0.45	0.35
3	0.33	0.27	1.00	0.18	0.20	0.29	0.19	0.18	0.38	0.25
4	0.23	0.28	0.18	1.00	0.36	0.32	0.36	0.20	0.26	0.23
5	0.33	0.54	0.20	0.36	1.00	0.43	0.85	0.09	0.38	0.34
6	0.45	0.47	0.29	0.32	0.43	1.00	0.38	0.15	0.46	0.25
7	0.28	0.48	0.19	0.36	0.85	0.38	1.00	0.10	0.37	0.35
8	0.15	0.12	0.18	0.20	0.09	0.15	0.10	1.00	0.15	0.16
9	0.55	0.45	0.38	0.26	0.38	0.46	0.37	0.15	1.00	0.26
10	0.24	0.35	0.25	0.23	0.34	0.25	0.35	0.16	0.26	1.00
11	0.27	0.30	0.27	0.25	0.28	0.29	0.29	0.18	0.26	0.34
12	0.17	0.16	0.19	0.22	0.14	0.19	0.11	0.20	0.15	0.18
13	0.23	0.20	0.21	0.24	0.18	0.20	0.17	0.35	0.16	0.24
14	0.30	0.21	0.27	0.26	0.21	0.30	0.19	0.16	0.25	0.19
15	0.28	0.25	0.30	0.19	0.22	0.26	0.20	0.20	0.21	0.27
16	0.31	0.30	0.28	0.26	0.30	0.34	0.28	0.17	0.24	0.27
17	0.50	0.37	0.44	0.27	0.37	0.43	0.35	0.18	0.62	0.29
18	0.47	0.44	0.36	0.28	0.39	0.53	0.37	0.14	0.61	0.26
19	0.19	0.22	0.23	0.32	0.23	0.23	0.21	0.30	0.17	0.31
20	0.21	0.22	0.21	0.27	0.27	0.21	0.25	0.28	0.19	0.34

[a] The similarity calculation was performed using Accord (Accelrys, San Diego, CA). Tanimoto coefficients were compared to the compound shown with close to 1.0 being more similar and close to 0.0 being less similar.

Table 2. Validation of the Self-Calibrated Quantitation Method (Diverse Compounds)

compd	W_1 (mg)	C_1 ($\times 10^{-4}$ M)	ϵ_{214} (M^{-1} cm^{-1})	W_2[a] (mg)	W_2'[b] (mg)	accuracy (%)
1	1.8	17.9	603	0.9	0.9	0
2	1.6	9.6	4287	1.6	1.5	6.3
3	1.2	10.2	2914	1.2	1.1	8.3
4	1.3	7.6	4273	1.4	1.7	21.4
5	1.7	4.8	7075	1.2	1.5	25.0
6	0.9	2.6	5566	1.6	1.7	6.3
7	1.4	6.8	7051	1.1	1.2	9.1
8	1.2	8.8	4855	1.4	1.5	7.1
9	1.8	13.1	889	1.8	1.9	5.6
10	1.7	8.0	8257	1.8	1.7	5.6
11	1.0	4.4	4358	1.0	1.0	0
12	1.3	5.4	10 958	1.3	1.2	7.7
13	1.4	8.4	6599	1.0	0.9	10.0
14	2.2	12.4	2886	1.2	1.2	0
15	2.0	12.1	5485	1.3	1.5	15.4
16	1.5	7.3	7133	1.9	1.9	0
17	1.2	8.2	7726	1.0	1.1	10.0
18	1.3	7.1	2869	1.1	1.2	9.1
19	1.8	3.8	7416	1.1	1.6	45.5
20	1.2	5.0	6549	1.0	1.2	20.0
av						10.6 ± 8.0

[a] Accurate compound weight. [b] Determination based on UV absorbance at 214 nm.

Table 3. Validation of the Self-Calibrated Quantification Method (Purified Library Compounds)

compd	W_1 (mg)	C_1 ($\times 10^{-4}$ M)	ϵ_{214} (M^{-1} cm^{-1})	W_2[a] (mg)	W_2'[b] (mg)	accuracy (%)
21	10.3	4.4	11 900	1.2	1.3	8.3
22	10.3	6.4	5895	1.3	1.0	23.1
23	10.3	4.6	8985	2.7	1.9	29.6
24	10.6	2.6	11 762	2.6	2.6	0
25	10.3	3.8	4973	1.6	1.6	0
26	10.5	3.7	11 151	2.1	1.7	19.0
27	10.3	3.6	13 000	1.3	1.0	23.1
28	10.2	3.8	11 760	1.2	1.1	8.3
29	10.8	3.5	13 588	2.6	2.4	7.7
30	10.6	2.4	26 195	1.4	1.3	7.1
31	11.0	3.6	5427	3.0	3.2	6.6
32	10.1	2.4	18 587	1.5	1.1	26.6
33	10.6	3.2	10 653	3.1	2.7	12.9
34	10.8	9.2	4649	1.2	1.1	8.3
35	10.8	6.9	7464	1.6	1.5	6.3
36	10.6	3.4	9830	1.8	1.5	16.7
37	10.4	3.0	15 227	1.0	0.8	20.0
38	10.9	3.2	10 225	1.1	1.0	9.1
39	10.1	2.9	6433	1.1	1.1	0
40	11.6	7.4	13 013	11.2	8.8	21.4
41	10.0	3.9	13 174	11.3	8.4	25.7
42	10.6	4.7	13 336	12.3	12.5	1.6
43	10.7	3.1	13 497	10.0	8.2	17.9
44	10.9	4.7	13 658	10.1	7.9	21.8
45	12.1	4.2	13 819	11.4	8.5	25.6
46	11.3	3.9	13 980	11.3	13.6	20.4
47	11.0	3.7	14 141	10.0	9.8	2.0
48	10.8	3.6	14 303	11.6	11.4	1.7
49	11.8	2.5	14 464	11.1	14.2	27.9
50	10.9	3.5	14 625	12.0	12.6	5.0
av						13.5 ± 11.0

[a] Accurate compound weight. [b] Determination based on UV absorbance at 214 nm.

calibration quantitation method using the extinction coefficients previously determined (Table 4). The compound loss is estimated to be 13−65%.

Although this method can address some general concerns regarding compound integrity over a long run, it cannot solve all issues at once. For example, (1) although the proposed method is generally applicable to all newly registered compounds, many legacy compounds were not purified or still have unknown purity. Repurifying these compounds is not a trivial task. Even the purity reanalysis, reweighing, and calculation of their extinction coefficient are a major undertaking. (2) The purified compounds normally have purities from 90% to 100%. A correction of the calculated extinction coefficient should be incorporated. (3) Some compounds may have insufficient solubility in standard solvent for HPLC sample preparation and yield incorrect extinction coefficents because of false concentration. A correct usage of solvent is crucial for quantitation. In certain cases, a separate quantitative measurement using quantitative detectors such as CLND may be necessary. (4) In a high-throughput analysis operation, it is difficult to maintain the ideal concentration for all compounds and entertain the highly parallel operation at the same time. Therefore, some reruns after dilution may be necessary. Hopefully, the problem samples may be identified by software so that the rerun list could be generated automatically.

A most urgent concern is whether this method still works when a different instrument is used in situations where (1) compounds are transferred to a different location, (2) the old instrument is replaced by a new one after many years, and (3) one needs to use several instruments to perform the analysis at the same time. Our solution to this is to use a group of reference compounds for calibration. We selected five compounds, **1**–**5**, as reference standards and measured their extinction coefficients in another instrument and

Table 4. Compound Loss Determination in a Simulated Compound Degradation Case

compd	$W_{initial}$ (mg)	$W_{degrade}$ (mg)	compd loss (mg)	loss (%)
21	10.3	6.9	3.4	33
22	10.3	7.5	2.8	27
23	10.3	3.8	6.5	63
24	10.6	6.0	4.6	43
25	10.3	4.7	5.6	54
26	10.5	8.0	2.5	24
27	10.3	5.8	4.5	44
28	10.2	4.3	5.9	58
29	10.8	8.6	2.2	20
30	10.6	3.7	6.9	65
31	11.0	8.1	2.9	26
32	10.1	4.4	5.7	56
33	10.6	7.8	2.8	26
34	10.8	9.4	1.4	13
35	10.8	6.5	4.3	40
36	10.6	5.4	5.2	49
37	10.4	5.0	5.4	52
38	10.9	5.8	5.1	47

Table 5. Cross-Instrument Calibration Using Calibration Standards

compd	W_1 (mg)	C_1 ($\times 10^{-4}$ M)	ϵ_{214}[a] (M^{-1} cm^{-1})	ϵ_{214}[b] (M^{-1} cm^{-1})	calibration constant
1	1.4	9.1	603	1195	0.5
2	1.6	10.4	4287	2501	1.7
3	1.7	9.2	2914	3746	0.8
4	1.4	8.3	4273	3352	1.3
5	1.3	7.2	7075	4750	1.5
av					1.1 ± 0.5

[a] Determined on instrument 1 (Shimadzu LCMS-2010EV equipped with a PDA detector). [b] Determined on instrument 2 (Waters 2795 separation module, Micromass ZQ detector, and Waters 2996 PDA detector).

Table 6. Quantitation Using Cross-Instrument Compared with Gravimetric Weight

compd	ϵ_{214}[a] (M^{-1} cm^{-1})	ϵ_{214}[b] (M^{-1} cm^{-1})	W_2 (mg)	W_2'[c] (mg)	accuracy (%)
1	603	548	2.1	1.6	23.8
2	4287	3897	2.4	1.9	20.8
3	2914	2649	2.5	2.9	16.0
4	4273	3885	2.1	1.5	28.6
5	7075	6432	1.9	1.1	42.1
6	5566	5060	2.0	0.8	60.0
7	7051	6410	1.8	0.8	55.6
8	4855	4414	1.4	0.9	35.7
10	8257	7506	1.6	1.3	18.8
12	10 958	9962	1.9	0.8	57.9
13	6599	5999	3.1	1.5	51.6
14	2886	2624	2.3	3.5	52.2
15	5485	4986	1.5	1.0	33.3
19	7416	6742	2.2	1.7	22.7
20	6549	5954	1.9	1.5	21.1
av					33.3 ± 15.8

[a] Determined on instrument 1. [b] Calibrated to instrument 2. [c] Determination based on absorbance measured on instrument 2.

calculated a calibration constant as 1.1 (Table 5). Using this calibration factor, we quantified 15 compounds using their original extinction coefficients. The results deviate from the weight determined gravimetrically by 33.3% (Table 6).

There are several immediate applications of this method: (1) quantitation of storage compounds at the time of screening to determine the real concentration for primary screening and IC_{50} measurements and (2) reanalysis of the compound collection by quantitation of compound loss caused by decomposition, freeze/thaw, and other effects during storage. There are also remaining challenges: (1) a computation platform is needed to automate data tracking for the purified compounds and the related calculations for quantitation, and (2) more cross-validation needs to be performed with a wide range of instruments, detectors, and over a time span.

In principle, detectors like ELSD or CAD can also be used for such quantitation using the self-calibrated quantitation principle and obtain comparable results. However, the UV detector is the single most widely distributed instrument, and a method using this instrument will have a more significant impact. Furthermore computational methods, such as the more reliable UV spectra prediction,[34] will be a further addition in this effort.

Acknowledgment. This work was supported by Shandong University, the American Lebanese and Syrian Associated Charities (ALSAC), and St. Jude Children's Research Hospital.

Supporting Information Available. Chemical structures of library compounds and selected LC/MS/UV_{214} data. This material is available free of charge via the Internet at http://pubs.acs.org.

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CC800007K