Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis 41 (2006) 152–157
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Evaluation of the chemiluminescent nitrogen detector for solubility determinations to support drug discovery

Shobha N. Bhattachar*, James A. Wesley, Christopher Seadeek

Pfizer Global Research and Development, Ann Arbor Laboratories, 2800 Plymouth Road, Ann Arbor, MI 48105, USA

Received 15 September 2005; received in revised form 4 November 2005; accepted 7 November 2005
Available online 20 December 2005

Abstract

Solubility measurements using chemiluminescent nitrogen detection (CLND) has advantages of reduced compound requirement and increased throughput compared to UV-spectrophotometric and HPLC-based measurements. CLND with direct flow injection was evaluated for the measurement of thermodynamic solubility to support drug discovery. The limit of quantitation (LOQ), accuracy, and day-to-day reproducibility of the detector were measured. Measurements made on CLND were compared to those obtained from UV spectrophotometry and HPLC. Based on the results obtained, it was concluded that the CLND performs satisfactorily for discovery stage thermodynamic solubility measurements.

Keywords: Chemiluminescent nitrogen detection; Solubility; Drug discovery

1. Introduction

Through the various phases of discovery and development, solubility information serves a wide range of needs. In the early stages, solubility information serves to characterize compounds belonging to a chemical series, and to determine if these compounds have sufficient solubility in the solvents employed in various activity screens. Typically, for the purpose of activity screens, kinetic solubility measurements are used. Kinetic solubility testing methodologies only require sub-milligram quantities of material and measurements can be made at a high throughput rate of up to 300–400 compounds per week with one instrument [1]. As compounds advance past the initial activity screens, the need for thermodynamic solubility data increases. Structure–solubility relationships, ADME profiling, and formulations for toxicology, pre-clinical and phase 1 clinical formulations all rely heavily on thermodynamic solubility data. Therefore, once crystalline material of reasonable purity becomes available, it is important to measure the thermodynamic solubility of compounds.

Conventional (traditional shake flask) methods of thermodynamic solubility determinations are either based on manual operations or are done on automated solubility workstations. These methods involve the stirring of excess compound in the solvent of interest over a period of several hours or days, followed by filtration of the saturated solution to remove excess undissolved solid, and analysis of the filtered solution by UV-spectrophotometric or HPLC analysis to determine the amount of dissolved compound in the solution. Automated workstations accomplish the same steps using powder dispensers, liquid handling systems and filtration units. Saturated solutions are prepared by adding compound and the desired aqueous solvent into sample tubes or deep well plates and agitating over several hours. The saturated solutions are then filtered through filter plates with the aid of vacuum or positive pressure. Standard solutions of each compound being tested are prepared by dissolving known amounts of compounds in the solvent. The process of solvation is usually assisted by the use of a small volume of cosolvent that is not UV-active. From these solutions of known concentration, a series of standard solutions are made by dilution. The standards and filtered saturated solutions are then analyzed either on a UV plate reader or by HPLC. Calibration curves for each compound are constructed from the analysis of the standards. Quantitation of the concentration in saturated solutions is based on the compound-specific calibration curves. The minimum compound requirement with these methods is approximately 5–10 mg. Typically, a single instrument can handle a throughput of 200 compounds per week.

* Corresponding author.
E-mail address: shobha.bhattachar@pfizer.com (S.N. Bhattachar).

0731-7085/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2005.11.009

Compared to manual operations with traditional shake flask methods, automated solubility workstations are both compound- and labor-sparing. However, there is a continued impetus in the industry to do more with less compound and time. To this end, the chemiluminescent nitrogen detector (CLND) appears to be an attractive option compared to spectroscopy-based instruments (such as UV and HPLC) for thermodynamic solubility determinations [2]. Unlike spectroscopy-based instruments that employ compound-specific calibration curves and need a UV chromophore for quantitation, the CLND measures the nitrogen content in the sample of interest. With the exception of compounds containing adjacent nitrogen, the response of the detector is equimolar with respect to nitrogen [3], across a wide range of concentrations. Therefore, using the signal from the nitrogen detector, and knowing the number of nitrogen per molecule for each of the compounds being analyzed, the concentration of the compound in the analyte solution can be determined using a generic nitrogen calibration curve. The CLND has been previously reported to be useful for automated analysis of compound libraries [2–6]. In comparison with other single calibrant quantification methods, such as evaporative light scattering detection (ELSD) and proton NMR, CLND has been reported to be comparable to proton NMR and better than ELSD in terms of accuracy and reproducibility [7]. It must be mentioned however, that in comparison to UV and ELSD, the CLND has been reported to be difficult to maintain as it is significantly more complex, expensive, and resource-dependent [6–8]. Additionally, compounds dissolved in solvents containing nitrogenous components are not amenable to analysis using CLND.

The CLND has been previously described in the literature [2,3]. The following is a brief overview. Fig. 1 shows a schematic of the CLND showing the different components of the detector. The sample enters the nitrogen detector, either via a direct flow injection or as an eluent from an HPLC column. It enters the pyrolysis tube of the detector through a nebulizer where it mixes with a blend of helium and oxygen to form a fine aerosol spray. In the pyrolysis tube, it is completely pyrolyzed at 1050 °C. The nitrogen in the sample is thus converted to nitric oxide. The nitric oxide then reacts with ozone to produce NO_2^*, which decays to the ground state with the liberation of photons. The number of photons liberated is proportional to the nitrogen content of the sample. It must be mentioned here that compounds that contain adjacent nitrogen (N–N, N=N and N≡N) are converted to molecular nitrogen (N_2) upon combustion. The CLND does not measure molecular nitrogen. For such compounds therefore, the CLND response is less than equimolar.

The objective of this work was to evaluate the performance of the CLND (LOQ, accuracy and day-to-day reproducibility) for the purpose of thermodynamic solubility measurements and to compare quantitation results obtained from this instrument with those from UV-spectrophotometric and HPLC instruments.

2. Experimental

2.1. Instrumentation

The Analiza ASOLW solubility workstation (Analiza Inc. Cleveland, OH) was used. The system incorporates the CLND as one of its main components. Mobile phase (75% methanol in water) was pumped through an HPLC pump into the nitrogen detector at a flow rate of 0.1 mL/min. Samples for analyses were drawn either from Mini-Uniprep vials or 96-well deep well plates via a Hamilton workstation. Samples were introduced into the CLND by direct flow injection. Oxygen and helium were used in the nitrogen detector at a flow rate of 60 ± 0.5 mL/min, monitored through digital pressure gauges installed on both gas lines. HPLC water was used to wash the injector probe and the sample loop between injections. The sample injection volume was fixed at 5 μL.

The following is a description of the ASOLW workstation [9]: the ASOLW program controls the experimental set up, the Hamilton workstation and parts of the nitrogen detector including zero adjustment and the electronics for scaling the photomultiplier gain. Proper adjustment of the sensitivity (gain) for each sample is essential in order to accurately measure nitrogen across a very wide range of concentrations. The correct sensitivity setting is achieved through 2–3 iterative steps, starting from

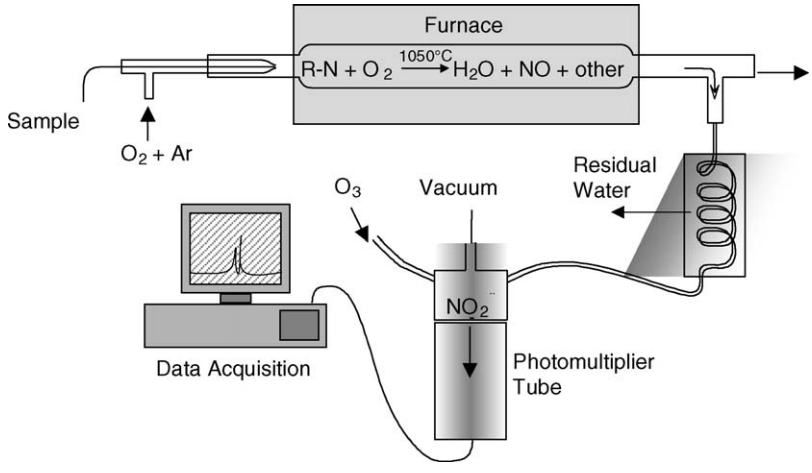

Fig. 1. Schematic showing the components of the chemiluminescent nitrogen detector. (Courtesy: Antek Instruments).

an initial injection at a default gain. The program also performs integration of the signals from the nitrogen detector.

Calibration is done using a calibration plate containing TRIS (Trizma base) solutions ranging approximately from 0.08 to 4300 ppm N. Each sample is injected in triplicate. Since the detector uses different sensitivity values to cover the large range of nitrogen concentrations, the actual instrument signal is always normalized as integral/sensitivity (I/S). These data, and the actual ppm N in each sample injected, are then used to generate a five-segment calibration curve. Each segment of the curve applies to a set range of I/S values and the corresponding ppm N concentrations. The first segment is fitted to a linear equation and the rest are fitted to exponential equations for ppm N concentration. The calibration curve thus generated is then stored in the configuration file of the ASOLW program.

When the assay is set up, the ASOLW program uses the molecular weight and number of nitrogen per molecule as input parameters for each compound. As each sample is run, and the raw I/S data are acquired, the program converts these values into dissolved sample concentration based on the calibration curve stored in the configuration file. Each sample run takes from 90 to 270 s depending on the number of iterations to optimize the integral value at an appropriate sensitivity setting.

2.2. Materials

Nitrogen detector calibration plates were either purchased from Analiza, Inc. or prepared in-house. Trizma base, atenolol, chlorpropamide, diltiazem hydrochloride, famotidine, furosemide, tolbutamide, norfloxacin, cimetidine, propranolol hydrochloride, nadolol, and verapamil hydrochloride were purchased from Sigma–Aldrich. All other compounds were obtained from Pfizer Compound Management.

2.3. Methods

The work that was carried out to evaluate the CLND performance may be divided into two parts: (1) evaluation of the limit of quantitation (LOQ), accuracy and reproducibility of the measurements; (2) comparison of CLND with an automated solubility workstation and manual HPLC measurements.

2.3.1. Calibration results and limit of quantitation (LOQ)

Calibration of the instrument was performed using calibration plates and the ASOLW calibration routine. The calibration curves were then obtained either by sending the raw data to Analiza for computation of the curves, or through a program written in-house that performed the computation in an identical manner.

Based on the reproducibility of the I/S values from triplicate injections of each nitrogen concentration in the calibration plate, the lowest concentration at which the % RSD from triplicate injections was less than 2% was determined from thirteen calibration curves. This concentration was defined as the practical LOQ of the system.

2.3.2. Accuracy of CLND analysis

The accuracy of CLND analysis was evaluated by analyzing several solutions of known concentration. Eleven compounds were evaluated. Two sets of sample solutions were prepared for each. The first set was a series of five standards and the second was a set of two samples. The standard solutions ranged in concentration from 10 to 100 μg/mL. The sample solutions were approximately 7.5 and 75 μg/mL. The compound concentration in each solution was calculated both from the generic nitrogen calibration curve and from a compound-specific calibration curve generated from the five standard solutions. The results were calculated as percent recovered of the known concentration for each sample.

2.3.3. Day-to-day reproducibility of the CLND results

The CLND requires regular calibration to correct for deviations from linearity at various sensitivity settings. All operating conditions remaining constant, the detector is expected to perform consistently over time. For the purpose of this work, Tris solutions, ranging in concentration from 1 to 1000 μg/mL were analyzed on two days, fifteen days apart. The reproducibility of the instrument performance was assessed by plotting the measured versus expected concentrations using the same generic calibration curve.

2.3.4. General comparison of CLND and traditional thermodynamic solubility measurements

The most attractive feature claimed for the CLND is its ability to measure thermodynamic solubility of compounds with reduced compound and time requirements compared to standard methods. The standard methods are based on UV spectrophotometry and HPLC analysis. UV spectrophotometry is most common in robotic solubility determinations. HPLC analysis is used where higher quality results are desired. HPLC has a low throughput and the quality of results is best when the analytical methods are optimized for the individual compounds.

Two separate sets of experiments were run – one to compare results obtained from an in-house UV spectrophotometry-based robotic solubility system (henceforth referred to as Robotic Solubility Determination System – RSDS) and another from HPLC analysis of a series of samples. For both the RSDS and the HPLC analyses, compound-specific standard curves were used for measurements. In addition, for the HPLC analysis, compound-specific methods were developed for accurate measurements. A total of 106 separate samples was used for the RSDS–CLND comparison and 111 samples for the HPLC–CLND comparison. For the RSDS samples, the nitrogen content ranged from 0.3 to 4.6 ppm N. This range is typical for compounds run on the system for high throughput thermodynamic solubility measurements. The nitrogen concentration for the HPLC samples ranged from 0.6 to 839 ppm. Considering that HPLC analysis is used for more complete solubility assessments of compounds such as pH-solubility profiles, this extended range of nitrogen concentrations is quite common.

3. Results and discussion

3.1. Calibration results and LOQ

The criterion for LOQ of the nitrogen detector was defined as the concentration at which the normalized detector response (I/S) resulted in triplicate injections having ≤2% RSD. The nitrogen concentrations measured in the calibration plates range from 0.08 to 4300 ppm N. Fig. 2 shows the mean % RSD obtained from all the experiments for the nitrogen concentration range of 0.1 to 8 ppm. These results clearly show that the LOQ for this instrument, according to our definition of the term, is approximately 1 ppm N. The relative standard deviations at each level of nitrogen concentration computed from 13 calibration experiments were found to be consistently <2% at nitrogen concentrations ≥1 ppm. Since the ASOLW has a fixed injection volume of 5 μL, this amounts to 5 ng of nitrogen. In terms of actual compound concentrations, for a compound with a molecular weight of 350 and 4 nitrogen per molecule, this would translate to a compound concentration of 6.25 μg/mL.

3.2. Accuracy of CLND results

The accuracy of the CLND measurements was assessed in two ways. First, for all of the standard and samples described in Section 2.3.2, the results obtained from the generic five-segment calibration curve, were compared to the known concentrations. The slope of this plot was 1.028 with an r^2 of 0.9928, meaning that the measured concentrations were 2.8% greater than the actual concentrations in the samples. It must be mentioned here that some of the lower concentration solutions, depending on their nitrogen content per molecule, were below the general LOQ of 1 ppm N. They were included nonetheless to evaluate the extent of deviations from expected values; compounds with solubilities in this range are quite common. Recognizing that conventional quantitative analysis is always done using compound-specific calibration curves, the results of the analysis were also computed from specific calibration curves for each compound. The slope of this comparison was 0.9974 with an r^2 of 0.9972. Fig. 3 summarizes the results from the generic nitrogen calibration curves compared with those obtained from the compound-specific calibration curve for the 11 compounds analyzed at different concentrations. The comparison shows that the generic nitrogen calibration curve is comparable to the compound-specific calibration curves.

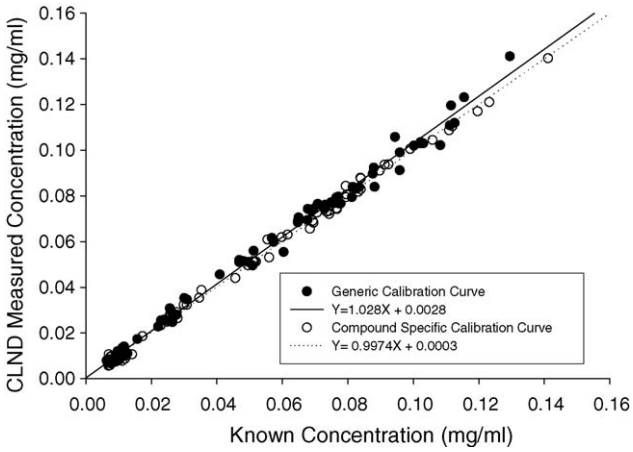

Fig. 3. Results obtained from generic calibration vs. compound-specific calibration ($n = 77$).

3.3. Day-to-day reproducibility of CLND results

Day-to-day reproducibility as assessed from the plot of expected versus measured Tris solutions, ranging in concentration from 1 to 1000 μg/mL is shown in Fig. 4. As seen from the plot, the data from the two sets of measurements, made fifteen days apart, were reproducible. This supports the proposition that the CLND, with all its operating parameters remaining constant, performs consistently over a period of at least fifteen days.

3.4. General comparison of CLND and traditional thermodynamic solubility measurements

The main goal of this work, as stated previously, was to compare the CLND with UV spectrophotometry-based instruments

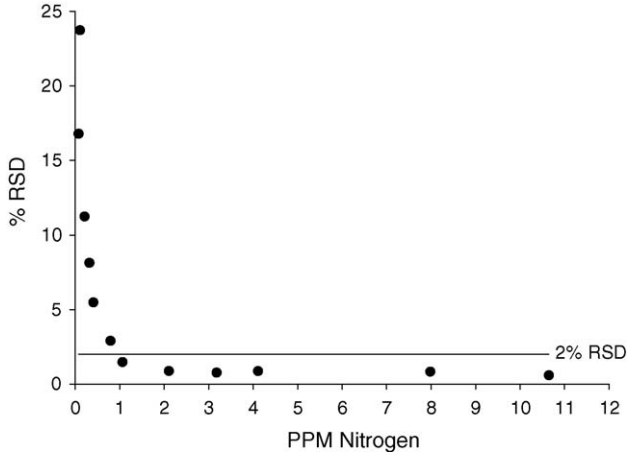

Fig. 2. Summary of reproducibility of instrument response vs. ppm N concentration ($n = 13$ for each data point).

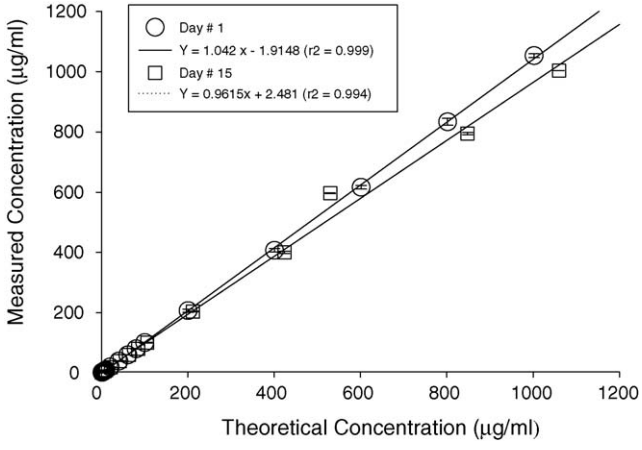

Fig. 4. Plot showing day-to-day reproducibility of CLND performance ($n = 3$ for each data point).

Table 1
Binning matrix analysis of Analiza and RSDS data

Solubility Binned by ASOLW values	Binning Concordance by # Compounds			
H (>60 μg/mL)	0	1	7	106 Compounds
M (10 -50 μg/mL)	2	33	2	
L (<10 μg/mL)	56	5	0	
	L (<10 μg/mL)	M (10 -50 μg/mL)	H (>50 μg/mL)	
	Solubility Binned by RSDS values			

for thermodynamic solubility measurements. Results of the comparison between 106 samples run on the CLND and RSDS are shown in Fig. 5. There was good agreement between the results obtained from the two instruments, as evidenced by a correlation coefficient of 0.92 and a slope of 0.996.

In early stages of discovery where high throughput is of essence and material is not always crystalline, it is sufficient to be able to assign compounds into low, medium and high solubility bins. Table 1 shows the binning matrix analysis between the CLND and RSDS data. As seen from the analysis, the results are concordant for 96 compounds tested, and within ±1 risk bin of each other for the rest of the 10 samples.

It is clear from this data that overall, the comparison between the CLND and RSDS systems is very good and that the CLND would be a good substitute for RSDS measurements.

HPLC measurements are considered the gold standard for quantitative analysis. Therefore, an additional comparison was made between HPLC and CLND results with a total of 106 samples from five compounds. The result of the comparison is shown in Fig. 6 below. The plot shows relatively good agreement between the HPLC and the CLND results.

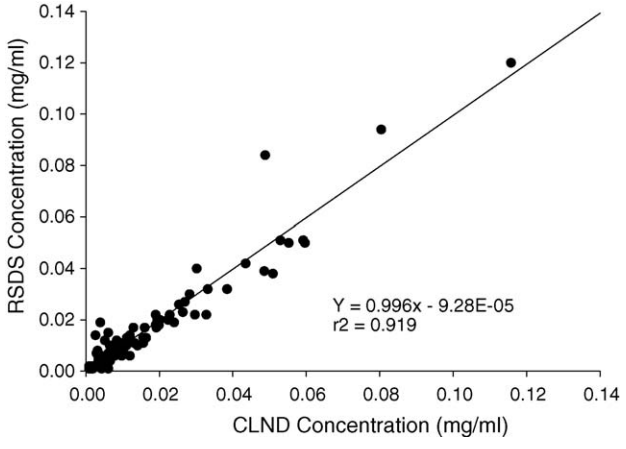

Fig. 5. Comparison of results obtained from CLND and RSDS systems ($n = 106$).

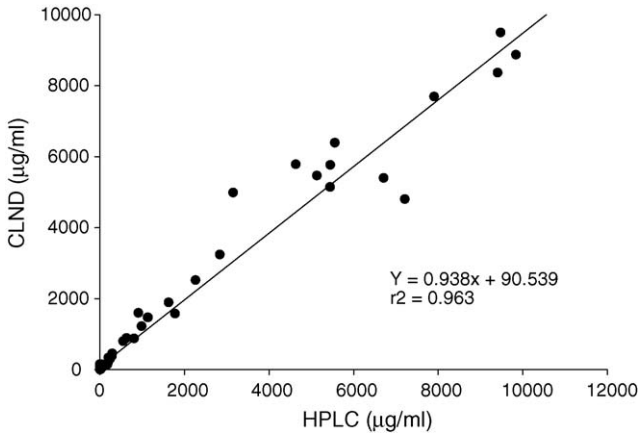

Fig. 6. Plot showing comparison between measurements made by HPLC and ASOLW for a total of 111 samples from five compounds.

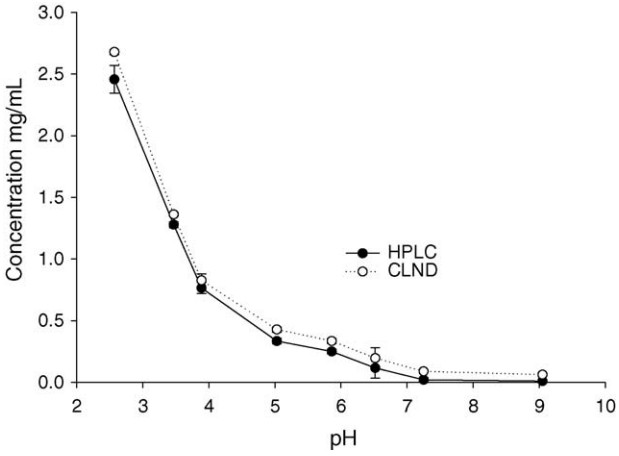

Fig. 7. Overlay of pH–solubility profiles of Pfizer compound A obtained from HPLC and CLND measurements ($n = 3$ for each data point).

In order to understand the practical implications of the differences between the two types of measurement, pH-solubility profiles of one of the test compounds (Pfizer compound A) obtained from CLND and HPLC analysis were overlaid. Equilibrium pH measurements were plotted against solubility values (Fig. 7). It is evident from the plot that in the context of pH solubility, all measurements are in excellent agreement.

4. Conclusions

This work shows that the CLND with direct flow injection is well suited for rapid quantitative analysis of single component samples of nitrogen-containing compounds in non-nitrogen containing solvents. The accuracy and reproducibility of the instrument is excellent above the nitrogen concentration of 1 ppm. The day-to-day reproducibility of the system over 15 days is good. While the level of accuracy of the CLND may not meet the stringent standards required for some highly precise quantitative analysis of pharmaceuticals (e.g. potency, stability and purity evaluations), it is sufficient for solubility determination, especially at early stages of research. The system produces

Table 2
Comparison between CLND, RSDS and HPLC methods for thermodynamic solubility measurement

System	Compound (mg)	Throughput	Range (μg/mL)	Other comments
Chemiluminescent N detection	3	96 Compds/8 h shift	Up to 6000	Compounds must contain N and no adjacent N[a]. No method development involved.
Robotic solubility (UV-spectrophotometry)	10	200 Compds/week	1–1000	UV chromophore required
Traditional with HPLC, UV detection	10	10 Compds/week	No limitations	UV chromophore required impurities and deg products resolved

[a] Compounds with adjacent nitrogen can be analyzed with compound specific calibration curves.

satisfactory measurements of solubility with a reduced compound consumption of approximately 3 mg. It has a throughput of 96 compounds per day.

As discussed earlier, in discovery stages where kinetic solubility does not meet all the project requirements, the CLND provides solubility data that is in relatively good agreement with RSDS and HPLC measurements.

Table 2 shows a comparison between the CLND, RSDS and HPLC methods in terms of compound requirement, throughput and range of solubilities measurable in a standard operating mode. It is clear from the comparison that the CLND offers the advantages of reduced compound requirement and increased speed over the other two types of instruments. However, CLND detector response relies on a number of variables that must be controlled for proper operation such as nebulizer performance, membrane dryer condition, gas flows and vacuum settings. Therefore, the knowledge and skill level needed to operate and maintain the CLND is greater than the more robust UV detectors used in the RSDS and HPLC systems. The increased proficiency required for the CLND is perhaps the largest hurdle to overcome in routinely utilizing the detector.

Acknowledgements

The authors would like to thank Julie Spence and Craig Wolfangel for analytical support.

References

[1] C. Lipinski, Methods Principles Med. Chem. 18 (Drug Bioavailability) (2003) 215–231.
[2] J-F.A. Borny, M.E. Homan, LC GC Europe 5 (1999) S14.
[3] K. Lewis, D. Phelps, A. Sefler, Am. Pharm. Rev. 3 (2000) 63–68.
[4] E.W. Taylor, M.G. Qian, G.D. Dollinger, Anal. Chem. 70 (1998) 3339–3347.
[5] W. Li, M. Piznik, K. Bowman, J. Babiak, Abstract of papers, 221st ACS National meeting April 2001.
[6] D. Corens, M. Carpentier, M. Schroven, L. Meerpoel, J. Chromatogr. A 1056 (2004) 67–75.
[7] S. Lane, B. Boughtflower, I. Mutton, C. Paterson, D. Farrant, N. Taylor, Z. Blaxill, C. Carmody, C.P. Borman, Anal. Chem. 77 (2005) 4354–4365.
[8] M. Styslo-Zalasik, W. Li, J. Pharm. Biomed. Anal. 37 (2005) 529–534.
[9] A. Chait, B.Y. Zaslavsky, PCT Int. WO 2003016883 (2003) 6–41.