THE FLUOROSOME® TECHNIQUE IN DRUG DISCOVERY

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News Flashes – The Fluorosome Company will display at the 2009 AAPS Workshop: Drug Transporters in ADME: From the Bench to the Bedside, Baltimore, MD, March 30-April 1.

GLSynthesis Inc. receives phase II SBIR grant to complete Fluorosome-trans-pgp development, p. 3.

United States Patent 7,060,292 "Lipid Structures and Their Utility" describing Fluorosomes issued on June 13, 2006.

Background

Large numbers of compounds are generated by combinatorial chemistry in the pharmaceutical industry for high-throughput screening assays. Methods to reduce the number of potential hits by determining properties that are predictive of therapeutic usefulness are in great demand. Many candidate drugs, for example, are ineffective because they cannot enter cells or cross the barriers that exist between various body compartments (e.g. intestinal, blood:brain barrier, placenta).

We offer a sensitive *in vitro* spectroscopic technique, the Fluorosome® Technique, that can address this and related issues in drug discovery. This technique measures rates of passive compound diffusion through true membranes (*permeabilities*) and active transport in the presence of transporters. These properties may be correlated with drug absorption and distribution and drug interactions *in vivo*. This technique is rapid, requires minimal amounts of compounds, and is amenable to robotics for high-throughput testing. Added advantages are that it uses no animals, cells, radiolabelled material or compound-specific detection methods. It is a valuable complement to existing tools used in the early screening of drugs.

Current membrane barrier models

Cell based methods. The Caco-2 cell line, derived from human colon adenocarcinoma cells, exhibits some characteristics of the small intestinal epithelial layer which represents a major barrier to gastrointestinal absorption. In this model system, membrane permeability coefficients are determined for the test compound by measuring compound transfer across a cell monolayer as a function of time. This model has advantages over animal testing – the cells are of human origin, results may provide good correlation to oral drug absorption in humans, and the method avoids the use of time-consuming, expensive and controversial animal studies.

In vitro methods. Cell-free permeability methods have been developed recently. IAM chromatography employs membrane bilayer analogs immobilized on HPLC columns. PAMPA (Parallel Artificial Membrane Permeation Assay) is a lipid/organic solvent based method, whose results correlate well with oral drug absorption. It is amenable to moderate throughput screening, and commercial robotic equipment is available to conduct the assays.

There are disadvantages to all current methods. Cell based assays require cell culture facilities and sterile conditions, and are limited to a narrow pH range. The above i*n vitro* methods do not utilize true membrane bilayers and lack flexibility. In all cases the analytical methods are compound-dependent, involving the use of radioactivity, suitable chromophores, or MS detection methods requiring expensive equipment. The assays are time consuming and not suitable for use in a high throughput screening program.

The Fluorosome-*trans* Technique

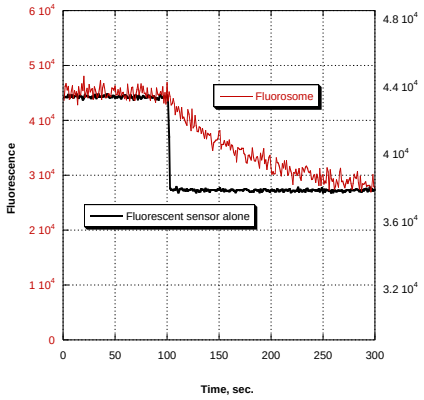
Fluorosome-*trans* is a type of Fluorosomes that is used tor measuring passive diffusion across membrane bilayers. The Fluorosome-*trans* Technique is a sensitive, rapid and universal *in vitro* method suitable for reliable measurement of drug permeabilities and for high throughput screening of compound libraries. The technique is applicable to drugs in solution or drug delivery vehicles over a wide pH range, and can be modified for specialized membranes and active transport processes.

The technique is based on measurement of the *rate of diffusion* of a compound through the membrane bilayer of a liposome and *detection* via quenching of the fluorescence signal of special fluorophores contained within the liposome. It can, thus, be run in any standard spectrofluorimeter. It is applicable to a wide range of molecules, and measures entry rates with half-lives from seconds to many minutes, corresponding to a wide range of permeability values. It has the advantages of employing no animals or living cells, does not require sterile conditions, and uses no radiolabel or compound-specific detection method. It is amenable to robotics and sufficiently sensitive to be used in multiwell plate spectrofluorimeters. Fluorosome-*trans* are manufactured by proprietary methods, and qualified with respect to unit fluorescence, particle size and permeability to test compounds. The Fluorosomes can be prediluted and stored at room temperature for 3 months.

The experiment is illustrated in Figure 1, which shows the fluorescence-time curve (red) for addition of a test compound to a Fluorosome suspension in a standard spectrofluorimeter at 100 sec. This time dependence is directly related to the rate of compound diffusion through the bilayer. (For example, the fluorescent probe in solution shows an immediate response to drug addition (Figure 1, black line).) The data are curve-fitted to give a first order rate constant k, which is used in conjunction

with the Fluorosome diameter to calculate the permeability, P, in cm/sec.

Figure 1. Fluorescence quenching of Fluorosome-*trans* and soluble fluorescent probe.

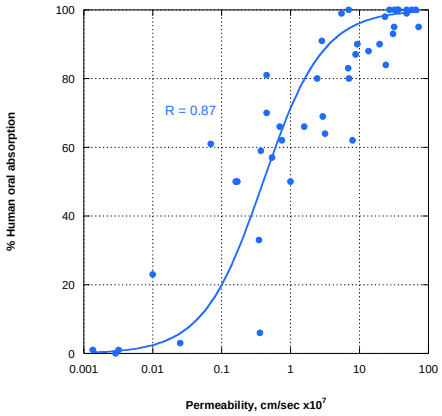


Fluorosome-*trans* assay validation

The validity of the method was first demonstrated by permeability assays with 44 marketed drugs of documented low, medium and high human oral absorption. The drugs have diverse structures and properties, are from a variety of therapeutic classes, and are believed to be absorbed by passive diffusion. Membrane permeability coefficients of these drugs were measured and compared with human oral absorption values (see the Appendix for data and literature references).

The permeability assays were done at 25 °C by addition of 20 µL aliquots of 50 mM solutions of drugs in DMSO to 2 mL of diluted Fluorosome-*trans* in a standard spectrofluorimeter. The time course of fluorescence change in each case was converted to the rate constant for diffusion of drug into the vesicles, and then used to calculate permeability. In all cases a clear, time-dependent change in fluorescence was observed. The magnitude of the change is different for each drug, but only the rate - not extent - of signal quenching, together with the particle diameter, are needed to calculate permeability coefficients, P. Values spanned a range of 10^{-6} to 10^{-11} cm/sec, and those for the 44 drugs analyzed were highly correlated with human oral absorption (Figure 2). The correlation follows a sigmoidal relationship similar to that first reported by Artursson and Karlsson comparing Caco-2 permeability with human oral absorption. The R value of 0.87 demonstrates the high correlation between P and oral absorption of the validation set of drugs.

Figure 2. Correlation between permeability measured with Fluorosome-*trans* and oral absorption for 44 drugs.



Published P values from Caco-2 and PAMPA assays for drugs in common with those of Figure 3 have been compared with the results for Fluorosome-*trans* (see Appendix). Caco-2 results for 27 drugs had a similar correlation (R = 0.73 vs. 0.67 for Fluorosomes), suggesting the reliability of Fluorosomes to mimic cellular membranes; however, Caco-2 data showed more outliers than Fluorosomes. Published PAMPA permeabilities varied widely, because assay conditions are different. For a common subset of 22 drugs reported by *p*ION Inc., the PAMPA results had more scatter and were more poorly correlated (R = 0.46 vs. 0.72 for Fluorosomes) than those obtained with Fluorosome-*trans*.

Few published results for drugs with low oral absorption are available. In consequence, data for PAMPA and Caco-2 P data are generally skewed to high absorption drugs (see Appendix). The extension of Fluorosome permeability values to poorly permeable drugs is possible because of the convenient time course of the assays (seconds to minutes), and the wide dynamic range of values (5 orders of magnitude) measurable by the technique.

Fluorosome-*trans*-pgp - NEW

The first of a series of active transport Fluorosome-*trans* is being developed with a SBIR grant from the National Institutes of Health. This "Fluorosome-*trans*-pgp" contains functional p-glycoprotein, aka MDR1, an important drug transporter, in the bilayer. GLSynthesis is completing development of assay procedures for inhibitors of pgp, and is working on assays for pgp substrates. Latest results will be presented at the 2009 AAPS Workshop: Drug Transporters in ADME: From the Bench to the Bedside, Baltimore, MD, March 30-April 1.

The Fluorosome® Solution, FS-1

The Fluorosome Company and Photon Technology International Inc. (PTI), Birmingham, NJ, have partnered to provide the FS-1, a package of hardware, software and reagents designed for drug permeability assays (Figure 3). PTI has custom-designed the FS1 as a high quality, inexpensive and small footprint spectrofluorimeter for Fluorosome®-*trans* and related assays.

Figure 3. Fluorosome Solution –FS-1.

The FS1 utilizes a solid state LED for excitation and a single photon PMT for detection, and special bandpass filters. With no lamp or grating, the FS-1 requires virtually no maintenance. The FS-1 contains features essential for Fluorosome® and related assays – a port for introduction of drug solutions to a cuvette containing Fluorosome-*trans*, a custom-designed autopipet with optical event marker, a magnetic stirrer for rapid mixing, and a thermostatted sample chamber for temperature control. Uses dipsosable cuvettes! Intuitive FS1.5 software, installed on the Toshiba laptop provided, controls data acqusition and analysis.

The FS-1 has completed beta-testing and is available for purchase. The package includes spectrometer, autopipet with optical event marker, laptop with

FS1.5 software installed, a box of disposable cuvettes, one Starter Kit (see below), and installation and technical support for one year. Stay tuned for updates!

Products and services

We offer permeability assays with Fluorosome-*trans*. Results of duplicate assays can be obtained on as little as 1 mg of most compounds. Turnaround time is ca. 1 week, and prices are competitive.

Starter Kits - The Fluorosome-*trans* Technique can be used in your laboratory, with any standard spectrofluorimeter giving output in Excel or ASCII formats. The instrument should have an injection port and cuvette stirring capability. We recommend, however, the FS-1, now available (see above)! Reagent starter kits for implementing Fluorosome permeability assays include 200 ml Fluorosome-*trans*, (sufficient for 100 assays in standard cuvettes), 100 µL test compounds A and B, data conversion software, QC lot analysis.

Full assay procedures, instrumentation requirements and data analysis software (Excel-based) are provided in Starter Kits sold separately. Additional Fluorosomes and buffers are available at attractive prices. Purchase of a starter kit entitles the buyer to up to 1 hour of email or telephone tech support. For Fluorosome-*trans*-pgp, please inquire.

The Fluorosome Solution - Includes FS-1spectrofluorimeter, autopipet with event marker, cables, disposable cuvettes, Toshiba laptop computer with FS1.5 software, Starter Kit, installation and training, one year warranty and full technical support and upgrades.

Consultation in lab setup, training and further technical support are available at extra cost.

The Fluorosome®-*trans* Technique:

- is a platform for measuring passive or active membrane permeabilities of drugs and drug candidates;
- correlates highly with oral absorption and is superior to current methods;
- is rapid and inexpensive;
- is available for implementation in your laboratory or as a service.

For further information visit us at:

www.fluorosome.com

For orders or inquiries, email:

info@fluorosome.com

or call 508 754-6700 and ask about Fluorosomes®

Welcome to our headquarters and R&D labs at One Innovation Drive, Worcester, MA 01605 USA.

Appendix. Reported human oral absorption data, permeability coefficients (P) measured with Fluorosome-*trans*,[a] and P values reported for Caco-2 and PAMPA assays.

			Permeability coefficient, cm/sec x 10^7		
#	**Drug**	**% human oral absorption (ref)[b]**	**Fluorosome-*trans***	**Caco-2[c]**	**PAMPA[d]**
1	Caffeine	100 (1)	27.5	308	12.0
2	Diazepam	100 (1)	6.98	710	
3	Indomethacin	100 (1)	65.3	204	3.0
4	Salicylic acid	100 (1)	35.4	220	0.2
5	Verapamil	100 (1)	48.3	694	394
6	Phenobarbital	100 (2)	36.7		
7	Imipramine	100 (1)	57.6	141	140.4
8	Theophylline	100 (1)	32.3		0.4
9	Antipyrine	100 (1)	11.1		
10	Propranolol	99 (1)	22.3	218	143
11	Prednisolone	99 (1)	5.52		
12	Warfarin	98 (1)	23.6	211	15.8
13	Metoprolol	95 (1)	72.4	237	4.1
14	Timolol	95 (1)	31.9	128	6.1
15	Alprenolol	93 (1)	30.8	405	
16	Hydrocortisone	91 (1)	2.84	215	19.4
17	Barbital	90 (3)	-		
18	Sulindac	90 (1)	59.0		
19	Quinine	90 (2)	9.34		
20	Nicotinic acid	88 (1)	13.60		
21	Pindolol	87 (1)	8.81	167	1.2
22	Acetylsalicylic acid	84 (1)	24.2	90.9	1.1
23	Procainamide	83 (2)	6.91		3.1
24	Quinidine	81 (1)	0.45	204	45.6
25	Acebutolol	80 (1)	7.07	5.1	0.3
26	Dexamethasone	80 (1)	-	125	
27	Guanabenz	80 (1)	2.43	209	8.9
28	Carbamazepine	70 (2)	0.45	228	64
29	Ciprofloxacin	69 (1)	2.95		
30	Dipyridamole	66 (4)	0.70		
31	Enalapril	66 (1)	1.58	23.1	5.2
32	Ranitidine	64 (1)	3.17	4.9	0.1
33	Terbutaline	62 (1)	0.75	4.7	5.0
34	Ampicillin	62 (1)	7.94	2.27	
35	Furosemide	61 (1)	0.07	6.1	0.1
36	Sulfasalazine	59 (1)	0.37	3.0	2.3
37	Nadolol	57 (1)	0.54	38.8	2.8
38	Amiloride	50 (1)	0.17		2.7
39	Atenolol	50 (1)	0.16	5.3	0.6
40	Etoposide	50 (1)	1.0		
41	Sulpiride	44 (1)	-		0.3
42	Ribavirin	33 (1)	0.35		
43	Acyclovir	23 (1)	0.10	2.5	0.4
44	Oxybutynin	6 (2)	0.36		
45	Cidofovir	3 (1)	0.025		
46	Ceftriaxone	1 (1)	0.0014		1.7
47	Streptomycin	1 (1)	0.003		
	Gentamycin	0 (1)	0.0029		

[a] At 25 ^{o}C. [b] (1) Zhao *et al.,* J. Pharm. Sci. 90:749-784, 2001. (2) Veber *et al.,* J. Med. Chem. 45:2615-2623 , 2002. (3) AHFS Drug Information, American Society of Health System Pharmacists, Bethseda, MD, pp. 2102-2106, 2003. (4) Drug Information for the Health Care Professional, 23 Ed., Micromedex, Greenwood Village, CO, pp. 1103-1104. 2003. [c] Yazdanian *et al.,* Pharm. Res. 15:1490-1494, 1998. Artursson and Karlsson, Biochem. Biophys. Res. Commun. 175:880-885, 1991. Mandagere *et al.,* J. Med. Chem. 45:304-311, 2002. Yee S. Pharm. Res. 14:763-766, 1997. Walter *et al.,* J. Pharm. Sci. 85:1070-1076, 1996. Zhu *et al*., Eur. J. Med. Chem. 37:399-407, 2002 [d] www.pion-inc.com and Sugano *et al.,* J. Biomol. Screen. 6:189-196, 2001.