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Article

High-Throughput Drug Stability Assessment via Biomimetic Metalloporphyrin Catalyzed Reactions Using Laser-Assisted Rapid Evaporative Ionization Mass Spectrometry (LA-REIMS)

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Abstract: It is an utmost importance to characterize drug candidates as early in the development pipeline as possible. Appropriate application of the emerging high throughput analytical techniques unlocks the opportunities to obtain experimental data for *in vitro* absorption, distribution, metabolism and elimination (ADME) profiling of large compound libraries which is crucial for selection of lead compounds. In an usual scenario, only one drug candidate will be selected from thousands of early compounds based on several stability and screening results. A high to medium throughput screening approaches could significantly accelerates the subsequent research and production phases. Traditionally, the analysis of metabolic stability assays heavily relies on high throughput LC-MS/MS techniques to meet with the lead profiling demands. In this study presented an innovative experimental approach that can offer a refreshing alternative. Laser Assisted Rapid Evaporative Ionization Mass Spectrometry (LA-REIMS) is a quick and efficient method for characterizing complex biological samples without laborious sample preparation and chemical separation. Therefore, using an automated LA-REIMS well plate reader as fast as 8 seconds per sample, we assessed the oxidative metabolic stability of active agents using biomimetic metalloporphyrin-based oxidative model reactions at various pH for commercially available drug substances and compared stability measurements with traditional HPLC-DAD-MS results.

Keywords: high-throughput screening; metabolic stability; ambient mass spectrometry; LA-REIMS; biomimetic metalloporphyrin; lead profiling; in vitro models; drug metabolism

1. Introduction

The rapid identification of compound with optimal pharmacokinetic characters plays a pivotal role in drug research. To achieve this, *in vitro* absorption, distribution, metabolism, and excretion (ADME) screening assays are extensively developed and employed. These assays generate data that proves to be predictive of a compound's pharmacokinetic properties in both preclinical species and humans.[1,2] During drug discovery process and lead optimization researchers face the challenge of conducting a multitude of experiments within a limited timeframe. High throughput screening (HTS) systems emerge as indispensable tools, enabling the evaluation of up to 100,000 individual compounds in just a few days or weeks. The extensive utilization of preclinical animals *in vivo* to execute these experiments raises not only the specter of exorbitant development costs but also the ethical quandary of aligning with the escalating societal and regulatory expectations regarding the

minimization of animal testing in pharmaceutical research.[3] In addition, taking and preparing samples from blood, urine or tissues is time-consuming, the resulting metabolites are in extremely low quantities and the biological matrix present is a confounding factor that can make analysis very difficult. However, information on the enzymatic transformations that the compounds under study undergo in the body is necessary for both the development and the authorization process. For this reason, there is an increasing focus on in vitro systems avoiding biological matrix in the development of pharmaceuticals to test the solubility and solution stability of drugs in simulated gastrointestinal fluid, the absorption of active substances in various epithelial cells membranes[4], as well as the metabolism of drugs.[5]

In vitro biomimetic models have the potential to provide support or even serve as alternatives to the established gold standard in vitro liver microsome based methods. In 1979, *Groves et al.*[6] first applied synthetic metalloporphyrins in biomimetic oxidation reactions. Using a meso-tetraphenylporphyrin iron(III) – iodosylbenzene system, they modeled hydroxylation and epoxidation reactions catalyzed by cytochrome P450 (CyP450) for simple alkanes and olefins in dichloromethane solution. Nowadays the utilization of metalloporphyrins as bioinspired oxidation catalysts is an evolving topic of research. By understanding the CyP450 mechanism of action, chemists have been able to successfully mimic several types of oxidation reactions using metalloporphyrins as catalysts.[7] Metalloporphyrins have also been employed in investigations of drug metabolism and the synthesis of metabolites, showcasing their versatility in biochemical research.[8,9] Recently, as a model system for drug metabolism metalloporphyrin have been utilized as magnetic nanoparticles,[10] liver-on-a-chip nanoparticles[11] and as an immobilized catalyst in a continuous flow packed bed reactors[10]

One crucial aspect influencing the activity of metalloporphyrins is their pH sensitivity. *Wolak et al.* examined the catalytic mechanism of meso-tetrakis(2,4,6-trimethyl-3-sulphonyloxyphenyl) porphyrin - iron(III) dihydrate (FeTPPS(2,4,6-Me)) in relation to the chemical properties of the medium. They found that pH significantly affects the selectivity and the rate of catalyst degradation[12]. FeTPPS(2,4,6-Me) porphyrin complex as a redox partner promotes homolytic O-O bond cleavage of peroxide bond containing oxidants when the pH < 3.5 and pH > 7, which is analogous to the mitochondrial one-electron oxidation within living bodies. Between pH 4 and 7, the heterolytic O-O bond cleavage is the thermodynamically favored mechanism. This process corresponds to the two electron oxidation reaction catalyzed by the microsomal CyP450 enzyme system, which also occurs physiologically.[13] Also a key features of the application of synthetic metalloporphyrin-based systems is the rapid degradation of the catalyst in homogeneous oxidative media. The rate of degradation is highly dependent on the structure, the reaction medium and the quality of the oxidant used.[14]

Advancements in label-free, high-throughput mass spectrometry (MS) analysis has significantly advanced the capabilities for screening compound libraries. Notably, ambient ionization techniques like Acoustic Mist Ionization (AMI, 2-3 samples/s),[15] Desorption Electrospray Ionization (DESI, 2.7 samples/s),[16] Electrospray Ionization (ESI, 0.4 samples/s), and Acoustic Droplet Ejection (ADE, 0.45 samples/s),[17] as well as conventional Matrix-Assisted Laser Desorption/Ionization (MALDI, 2.5 samples/s)[18] and hybrid techniques such as Infrared Matrix-Assisted Laser Desorption Electrospray Ionization (IR-MALDESI 0.5-1.3 samples/s),[19] have shown promising results in terms of analytical speed. Despite their clear advantages over traditional label-based photometric methods, fast MS-based analysis applications remain relatively underutilized.

Rapid Evaporative Ionization Mass Spectrometry (REIMS) is an ambient ionization technique, initially pioneered for use in clinical environment for the real-time classification of biological samples and human tissue types.[20] Its ability to translate molecular information of mobilized sample aerosol to mass spectral data points offers desirable solutions for a wider range of applications. With laser powered sample mobilization and (OPO; Optical Parametric Oscillator laser source) easier to automate sampling devices transferred the REIMS technique into an advanced, automated sampling platform. LA-REIMS (laser assisted-REIMS) has been already used in multiple scientific disciplines such as salivary metabolomics,[21] microbiology,[22] metabolic biofluid phenotyping,[22] and food

authentication. [22] LA-REIMS can be used as a metabolomics platform in cervical cancer screening.[23]

Here, in this study we report the first early-stage drug research related application of automated and laser-assisted-REIMS (LA-REIMS) platform, which allows high-throughput analysis of CYP450-derived metabolic stability of drug substances using synthetic metalloporphyrin-based biomimetic oxidation reactions. LA-REIMS can also allow users to monitor in real time drug consumption and metabolite formation.

2. Materials and Methods

Materials

Analytical grade chemicals were used in the experiments. Methanol, acetonitrile, acetic acid, sodium dihydrogen phosphate, sodium hydrogen phosphate, disodium hydrogen phosphate, *tert*-butyl hydroperoxide (*t*BuOOH) as co-oxidizing agent for metalloporphyrin and drug substrates were purchased from Merck Kft (Budapest, Hungary). The catalyst mezotetrakis-(sulfonyloxyphenyl)iron porphyrin (FeTPPS) was purchased from Frontier Scientific Inc (Utah, USA). The formic acid used for analytical measurements was purchased from Alfa Aesar (Ward Hill, Massachusetts, USA). The water used for the measurements was produced using a Milli-Q water purification system (Millipore, Bedford, Massachusetts, USA).

LA-REIMS Well Plate Reader

For this work we utilized a custom-made LA-REIMS platform prototype which is designed for sampling from well plates. This LA-REIMS prototype is for research purpose only. The LA-REIMS technique is based on a direct analysis of the aerosol generated by laser ablation (OPOTEK OPO laser, $\lambda=2940\text{nm}$, 5mJ/pulse , 20Hz shot frequency, 6ns pulse width). The generated droplets were introduced into the REIMS atmospheric interface where the ion generation occurs via collision with a high temperature impactor surface. For focusing the ablation beam uncoated spheric calcium fluoride lens with 50mm focal length were used. Motorized 3D stages (Thorlabs, Newton, New Jersey, USA) has been used for positioning the well plates in X-Y-Z directions. Raw data was acquired by using a XevoTM G2-XS TOF-MS (Waters Corporation; Milford, MA, USA), the spectra were acquired in positive ion mode over the mass to charge range $50\text{-}1200$ with 0.5 sec/scans . Leucine enkephalin was used as a lock mass material. Data was processed with Waters MassLynxTM 4.2 software.

HPLC-DAD-MS Measurements

The HPLC-DAD-MS experiments were performed using a Waters MicromassTM Quattro Ultima Pt tandem quadrupole mass spectrometer coupled to a Waters 2795 liquid chromatography system (Milford, MA, USA). Detection was performed with a Waters 2487 Dual λ Absorbance detector. The analysis was performed at $40\text{ }^{\circ}\text{C}$ on a Kinetex XB C18 column ($150\times 4.6\text{ mm}$, $2.6\text{ }\mu\text{m}$) (Phenomenex, Torrance, CA, USA) at a mobile phase flow rate of 1 ml/min . The eluent A composition used was $0.1\text{ vol \%}$ formic acid dissolved in water and eluent B was a $95:5$ mixture of MeCN:H₂O to which $0.1\text{ vol \%}$ formic acid was added. The gradient elution program: a linear gradient of $5\text{-}100\%$ B was applied in the range $0\text{-}11\text{ min}$, and an isocratic phase of 100% B was applied in the range $11\text{-}13\text{ min}$. This was followed by a 2-min equilibration period before the next injection using a 5% B composition. The injection volume was adjusted to $5\text{ }\mu\text{l}$ and the chromatographic profile was recorded at a wavelength of $220 (\pm 4)\text{ nm}$. The mass spectrometry conditions were as follows: ion source ESI, positive ion mode, scanning ion mode ($150\text{ - }600\text{ m/z}$), drying gas (N₂) temperature $350\text{ }^{\circ}\text{C}$, flow rate 5.5 l/min , nebulizer gas (N₂) pressure 6 bar , quadrupole temperature $120\text{ }^{\circ}\text{C}$, capillary voltage 2500 V , fragmentor voltage 60 V . Data were processed using MassLynxTM 4.0 software.

3. Results

We have studied the biomimetic stability for 77 drugs on 3 pH level. The experiments were performed in 1.5mL microcentrifuge tubes ((Eppendorf® Safe-Lock). The total volume of each reaction mixtures and blanks was 0.5 mL. For the reaction mixtures 370 μ L of methanol, 10 μ L of 10 mM drug stock solution has been transferred into each tube. To the biomimetic samples it was added 13.6 μ L of FeTPPS (0.735 mM, dissolved in methanol) solution, then to the pH-dependent oxidizing agent-only and biomimetic samples were added 100 μ L of aqueous buffer solution (64 mM) of appropriate pHs (pH 2.7: formic acid, pH 4.5: acetic acid with sodium acetate; pH 7.4: monosodium- and disodium-phosphate). To the reaction mixtures it was added 6.8 μ L of *t*BuOOH stock solution (147 mM). The concentrations of the stock solutions and porphyrin solution are adjusted so that the proportions of each component in the final reaction mixture were 1:10:100 as the porphyrin:substrate:*t*BuOOH. For each drug substrate we prepared 10 samples and the final concentration for each drug were 200 μ M in each sample tube. The 10 samples were: 1 sample of standard dissolved in methanol (QC sample), which served as an additional reference for mass accuracy. Blank reaction solutions were also measured at 3 pH levels: pH 2.7, pH 4.5, and pH 7.4. Blank reaction solutions were, contained all components except for the metalloporphyrin catalyst. Biomimetic metabolism reactions were also conducted at 3 pH levels: Two parallel reaction solutions were prepared for each biomimetic reaction solution (parallel reaction mixtures). The reaction solutions contained the drug substance, the oxidizing agent (tert-butyl hydroperoxide), the metalloporphyrin catalyst, and the necessary buffers. The reason for conducting reactions at three different pH levels is to explore the pH dependency and optimal conditions of the metalloporphyrin catalyst behavior, which was mentioned in the introduction. Our results reflected that the activity of the applied metalloporphyrin catalyst is pH dependent.

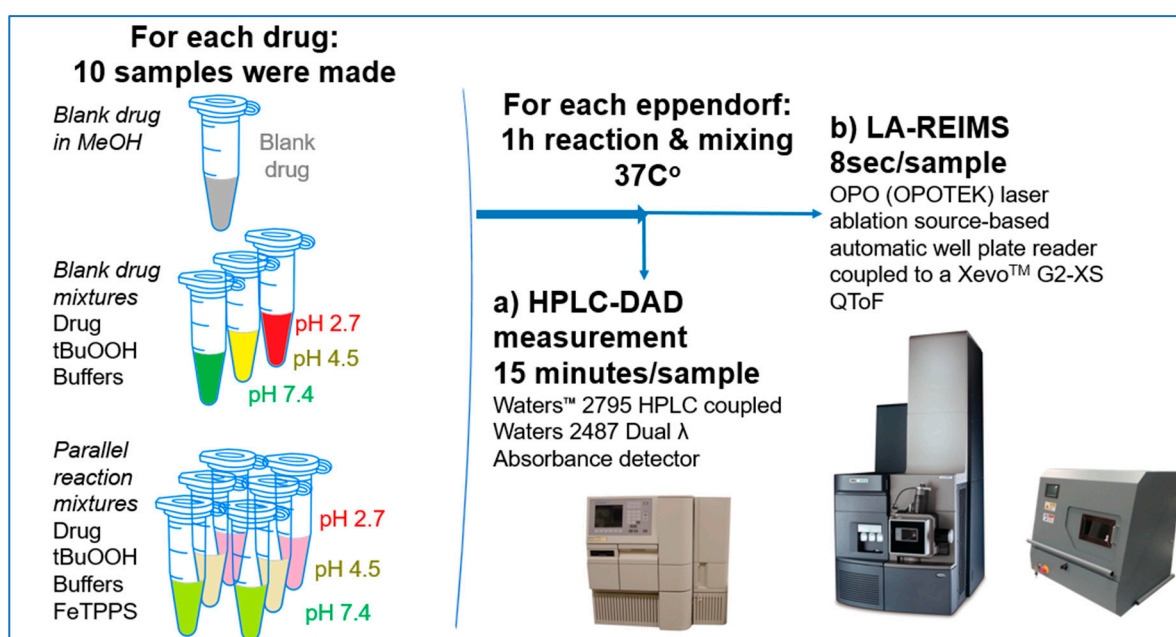


Figure 1. Experimental workflow of the high-throughput drug stability assessment

The prepared solutions were pipetted into the well plate, each row contains all of solutions from each sample (Figure 2.). Each well contains 100 μ L of solution, however during the study it was measured that we could use routinely 30 μ L sample volumes too. Data acquisition was started row by row, with one raw file containing all the ten datapoint of a given active pharmaceutical ingredient.

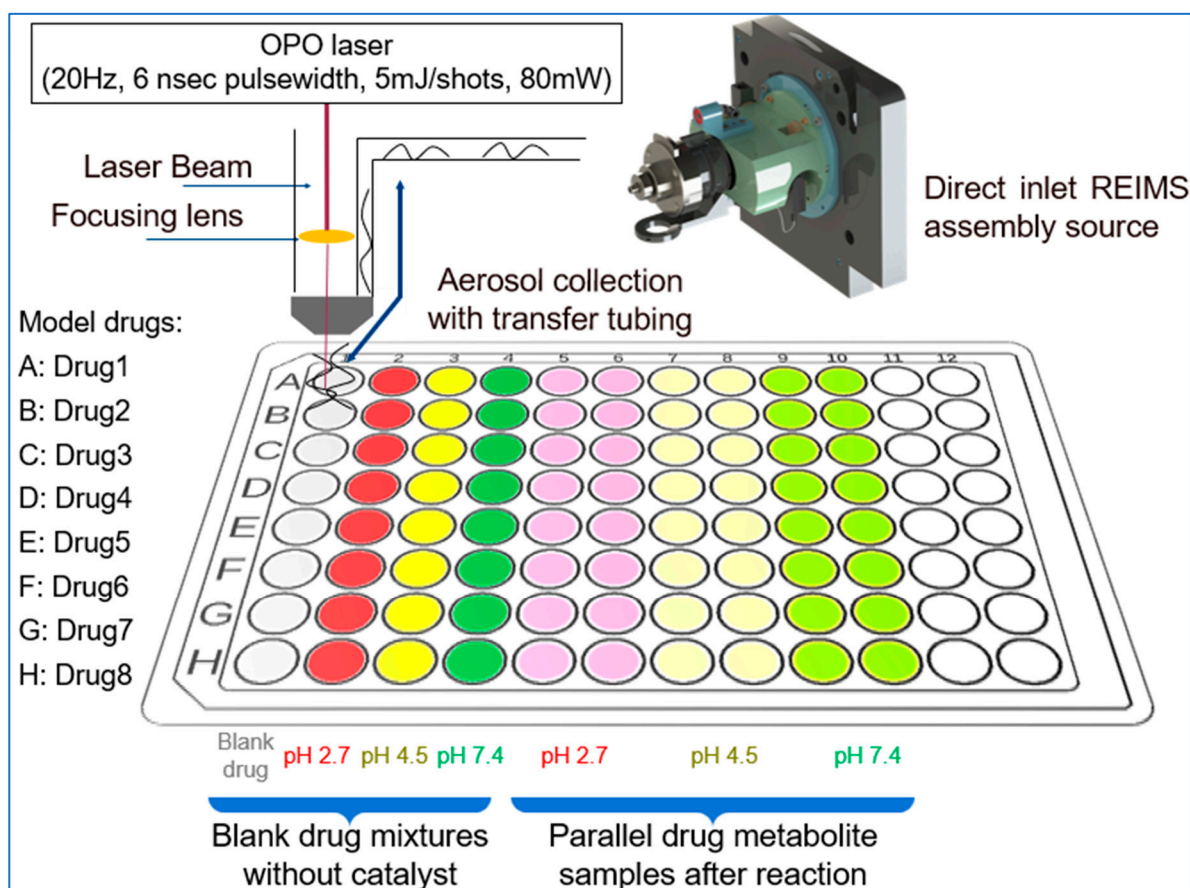


Figure 2. Scheme of the LA-REIMS measurement setup

Here below, on Figure 3 one can see the results obtained on the example of aripiprazole. The chromatogram below shows that the acquisition of the ten independent datapoint required only 1.5minutes. It is clearly visible from the ion chromatogram of aripiprazole (second trace from the top) that the degradation is close to complete in pH2.7, while in pH4.5 and pH 7.4 the degradation is not significant. The appearance of the major degradation products can also be followed as it is illustrated on the bottom three traces in Figure 3. To obtain these results for this compound would have taken 2.5 hours with a conventional HPLC-UV-MS.

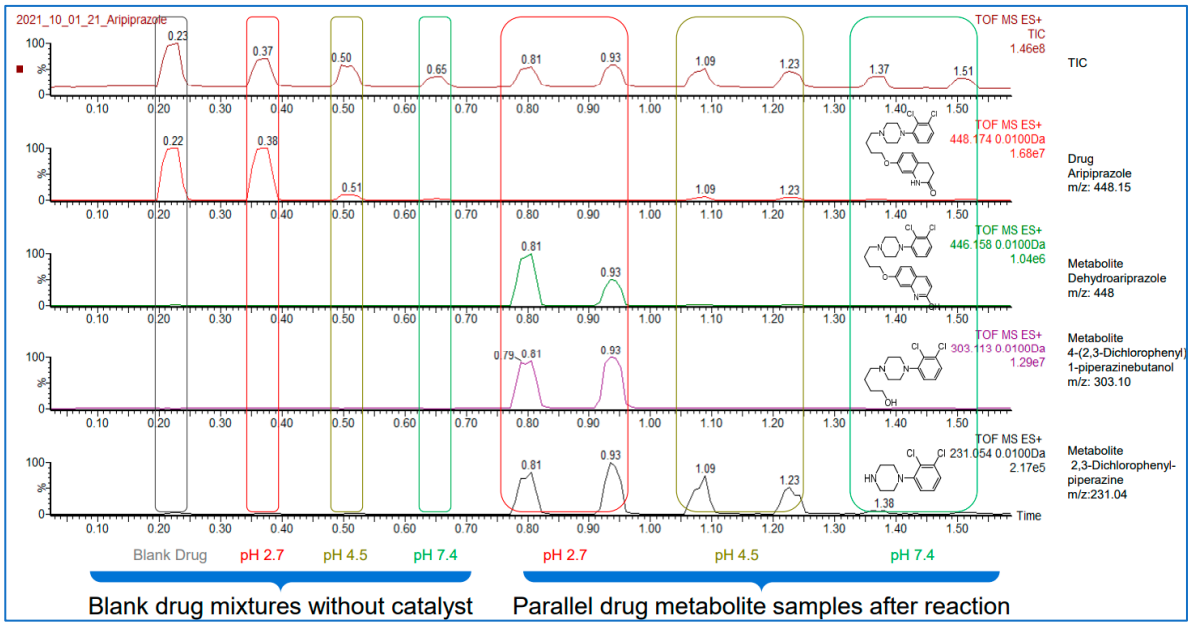


Figure 3. Example of the LA-REIMS acquired data. Chromatograms of the 10 samples of Aripiprazole model drug were acquired within 1.5 minutes. User can extract the required peaks and display them as an extracted chromatogram. pH dependency of the metabolite formulation and oxidative metabolism is visible from the chromatograms (TIC: total ion chromatogram).

3.2. Regression Comparison of Results Obtained Using LA-REIMS and LC-UV-MS

Conversion rates are calculated by the loss percent of the peak integral of the blank drug’s M+ compared to the drug’s M+ post-reaction value at their corresponding pHs. Good agreement is visible at the endpoints of drug conversion in the LC-DAD vs LA-REIMS plot on pH2.7 and pH4.5 after we omitted 10 and 3 outliers because of sample quality and instrument settings (Figure 5). Due to the matrix effect of buffers caused ion suppression at the detection, the sensitivity of LA-REIMS is reduced by orders of magnitude, especially on pH7.4. However, this occurrence is acceptable as beyond pH 7, the formation of the artificial oxo-dimer takes place, resulting in the deactivation of the metalloporphyrin catalyst.

In general, the results obtained with the LA REIMS workstation are nicely correlates with the conventional HPLC results, while required significantly less analytical runtime.

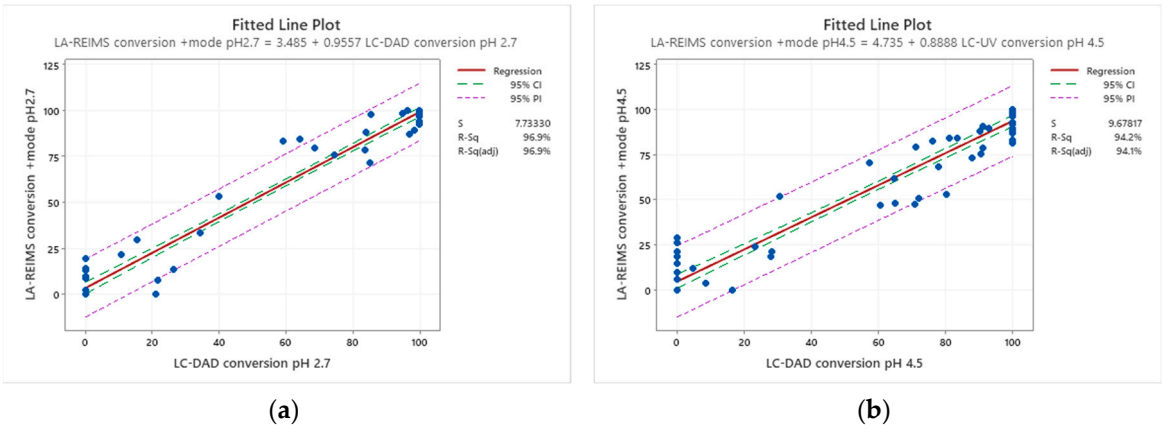


Figure 5. Regression plot on (a) pH2.7 and (b) pH4.5 in positive ion mode (67 and 74 model drugs from 77 drugs).

3.2. Solvent Requirements and Measurement Time

The high throughput screening capable LA-REIMS technology can be a greener alternative compared to traditional LC-based techniques due to its lower solvent requirement. While conventional liquid chromatography (LC) methods often demand significant amounts of solvents for analytical procedures, LA-REIMS technology consumes less solvent. This reduction in solvent usage not only minimizes environmental impact but also contributes to cost-effectiveness and sustainability. By requiring lower volume of solvents, LA-REIMS facilitates an eco-friendlier approach in analytical chemistry, aligning with the growing emphasis on green chemistry practices. Consequently, its integration into high throughput screening processes not only enhances efficiency but also underscores its role in advancing environmentally conscious analytical methodologies.

Table 1. Comparison of solvent requirements and measurement times of measurement techniques (* UHPLC results are from the literature, presented for comparability[24])

Technique	One sample	10 sample (to characterize a drug)	770 sample (whole dataset)
HPLC based	15 ml	150 ml	11,550 ml
	15 min	150 min	212.5 h
UHPLC based*	0.3 ml	3 ml	231 ml
	1 min	10 min	~13 h
HTS LA-REIMS	0.02 ml	0.2 ml	15.4 ml
	8 sec	80 sec	< 1 h 45 min

4. Discussion

In conclusion, LA-REIMS emerges as a promising technique, demonstrating potential suitability for semi-quantitative high-throughput metabolic stability in an optimized solvent environment. Notably, LA-REIMS possesses a distinct speed advantage over conventional HPLC measurements, without compromising sensitivity or incurring significant information loss. Reduced measurement time and the use of the LA-REIMS technique also reduces solvent requirements by orders of magnitude. LA-REIMS is thus a greener, more environmentally friendly measurement technique that is ideally suited to the modern pharmaceutical industry. The LA-REIMS can also facilitate the real-time recording of pharmacokinetic curves directly from a well plates, eliminating the need for sample preparation, which can also support the traditional ADME microsomal metabolic stability research. Our future endeavors aim comprehensive exploration of microsomal systems, leveraging upon this methodology.

Author Contributions: Conceptualization, Tamás Karancsi and György Tibor Balogh; Data curation, András Marton, Zsombor Mohácsi, Balázs Csillag, Júlia Balogh, Richárd Schäffer and Tamás Karancsi; Formal analysis, Zsombor Mohácsi, Balázs Decsi and Balázs Csillag; Funding acquisition, György Tibor Balogh; Investigation, András Marton, Zsombor Mohácsi, Balázs Decsi, Balázs Csillag and Richárd Schäffer; Methodology, András Marton, Zsombor Mohácsi, Balázs Decsi, Júlia Balogh and Richárd Schäffer; Project administration, Tamás Karancsi and György Tibor Balogh; Resources, Tamás Karancsi and György Tibor Balogh; Software, Tamás Karancsi; Supervision, Tamás Karancsi and György Tibor Balogh; Validation, András Marton; Writing – original draft, András Marton; Writing – review & editing, Júlia Balogh, Richárd Schäffer, Tamás Karancsi and György Tibor Balogh.

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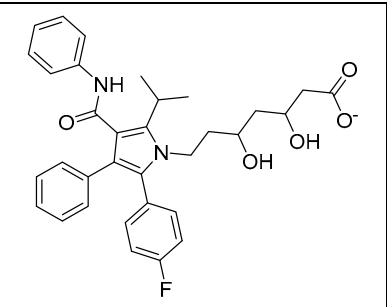
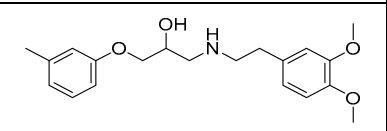
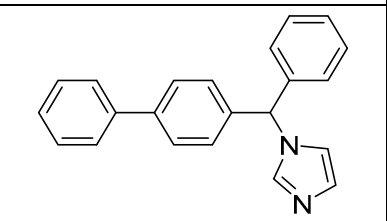
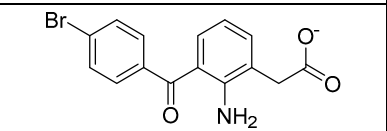
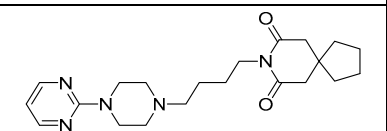
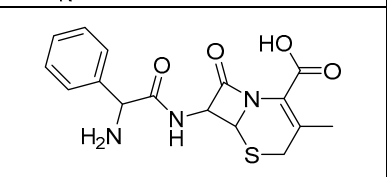
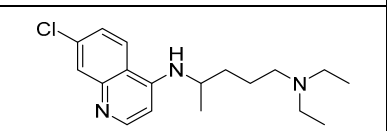
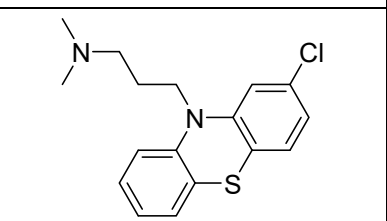
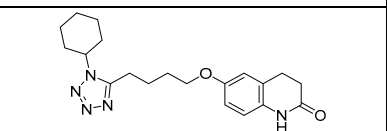
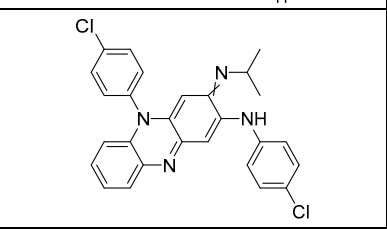
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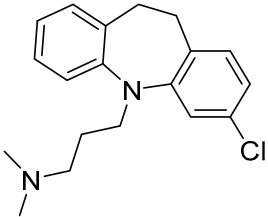
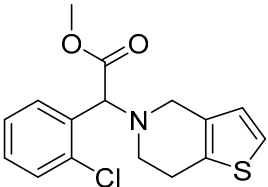
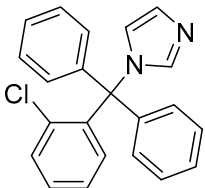
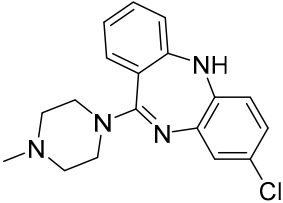
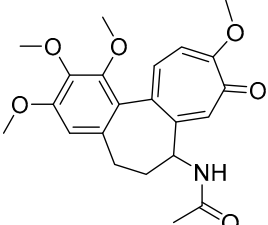
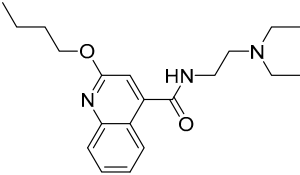
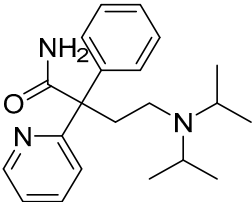
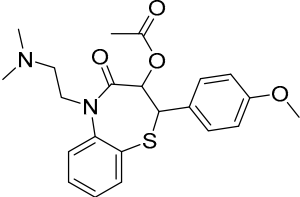
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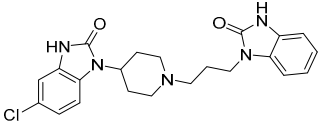
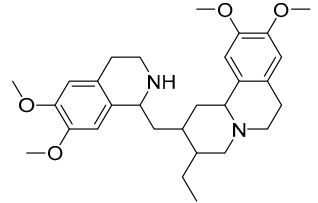
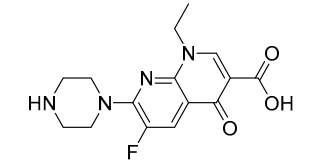
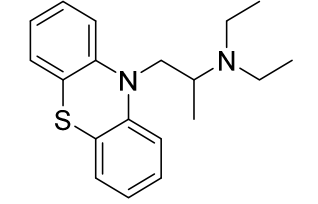
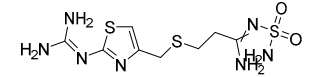
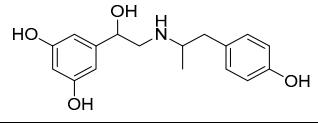
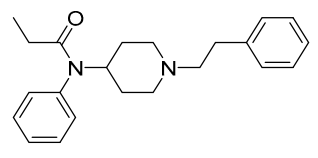
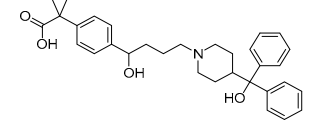
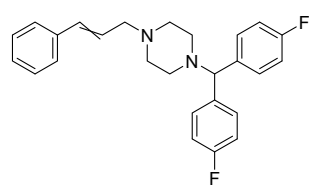
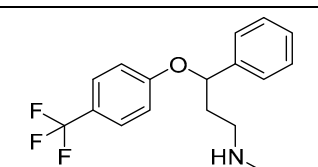
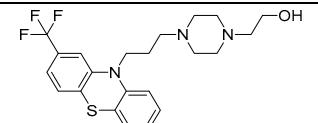
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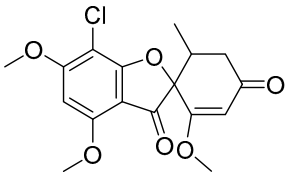
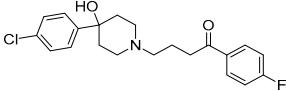
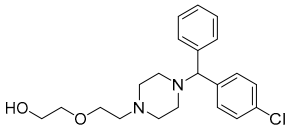
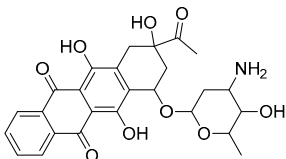
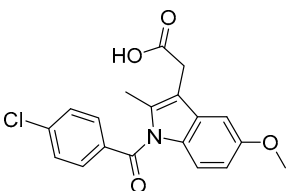
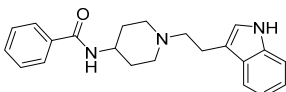
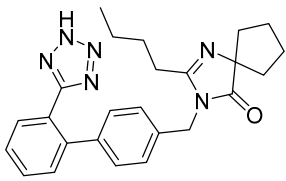
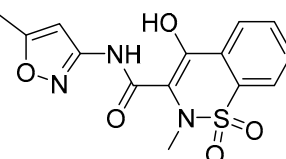
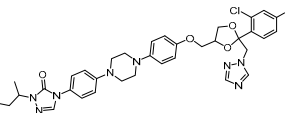
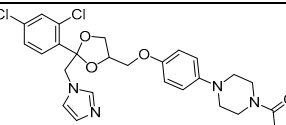
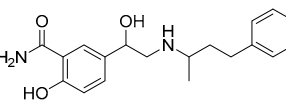
Appendix A

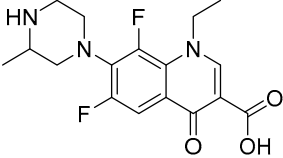
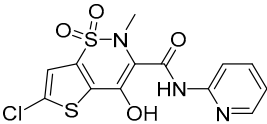
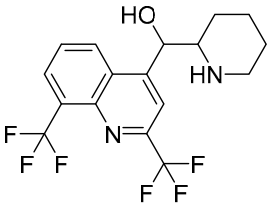
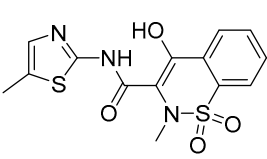
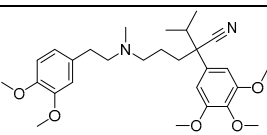
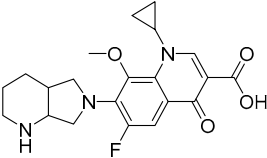
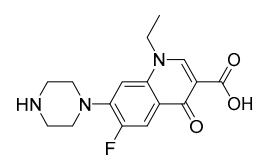
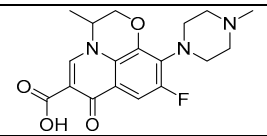
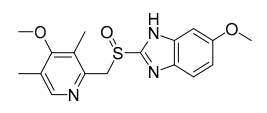
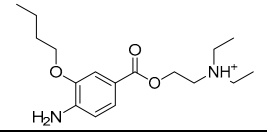
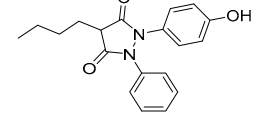
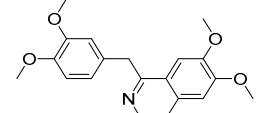
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			HPLC DAD	LA-REIMS	HPLC DAD	LA-REIMS
1	Amethopterin (Methotrexate)		100	97	100	Not detectable
2	Amodiaquine		100	100	100	99
3	Amoxicillin		84	79	100	Not detectable
4	Aripiprazole		100	100	99	52
5	Astemizole		96	100	100	100
6	Atorvastatin		100	92	100	83
7	Benidipin		100	94	100	98

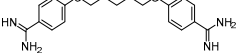
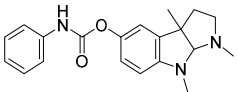
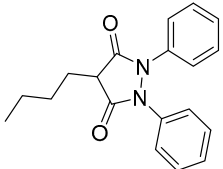
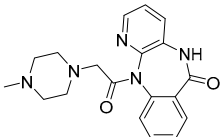
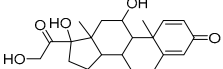
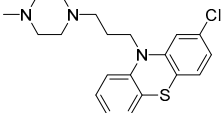
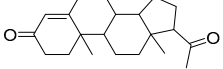
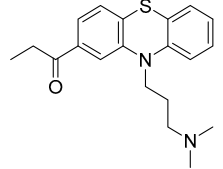
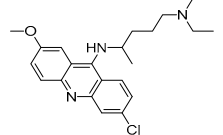
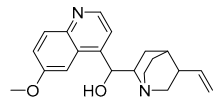
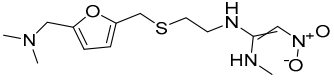
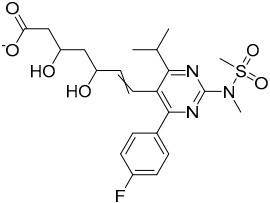
8	Bepridil		100	100	100	99
9	Bevantolol		40	0	53	29
10	Bifonazol		0	0	0	0
11	Bromfenac		100	100	Not detectable	100
12	Buspirone		54	78	No detection	100
13	L-Cephalexin		84	17	88	ND
14	Chlroroquine		11	24	57	96
15	Chlorpromazine		100	99	71	79
16	Cilostazol		95	98	65	62
17	Chlofazimine		100	100	88	73

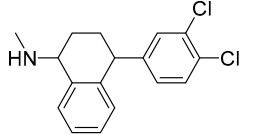
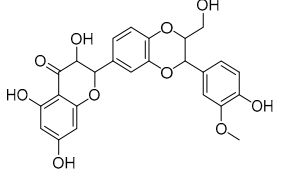
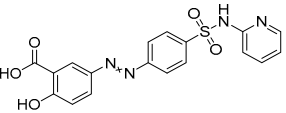
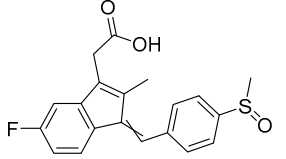
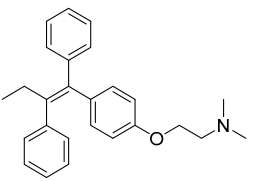
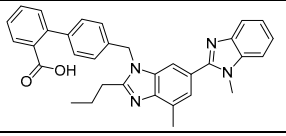
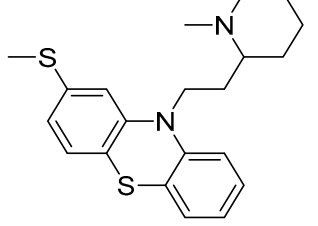
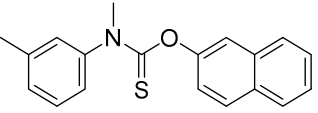
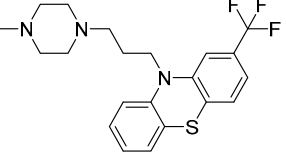
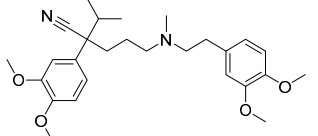
18	Clomipramine		60	99	81	84
19	Clopidogrel		26	13	23	24
20	Clotrimazole		0	0	ND	ND
21	Clozapin		100	99	93	90
22	Colchicine		0	0	90	15
23	Dibucaine		0	80	0	53
24	Disopyramide		0	5	0	12
25	Diltiazem		64	84	57	70

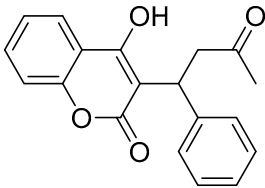
26	Domperidon		97	87	90	75
27	Emetan		100	97	76	83
28	Enoxacin		0	18	10	67
29	Ethopropazin (Profenamin)		100	99	100	93
30	Famotidin		100	99	100	92
31	Fenoterol		100	100	100	100
32	Fentanyl		0	0	0	21
33	Fexofenadin		22	8	28	19
34	Flunarizin		0	71	8	51
35	Fluoxetine		0	0	2	0
36	Fluphenazine		100	97	100	90

37	Griseofulvin		0	14	0	0
38	Haloperidol		0	13	31	52
39	Hydroxyzine		0	0	8	4
40	Idarubicin		100	99	100	100
41	Indomethacin		100	94	52	ND
42	Indoramin		100	100	100	100
43	Irbesartan		0	0	0	0
44	Isoxicam		70	0	100	100
45	Itraconazole		100	100	73	87
46	Ketoconazole		100	99	100	99
47	Labetalol		75	83	76	84

48	Lomefloxacin		0	0	0	19
49	Lornoxicam		100	99	100	99
50	Mefloquine		0	0	0	0
51	Meloxicam		57	97	100	97
52	Gallopamil		100	98	100	82
53	Moxifloxacin		100	99	28	21
54	Norfloxacin		9	19	38	45
55	Ofloxacin		29	100	17	0
56	Omeprazole		53	100	ND	ND
57	Oxybrupocaine		100	100	100	90
58	Oxyphenbutazone		100	100	100	87
59	Papaverine		15	29	0	0

60	Pentamidine		0	0	0	0
61	Phenserine (Fenszerin?)		100	62	100	92
62	Phenylbutazone		99	89	90	88
63	Pirenzepine		0	0	0	0
64	Prendisoline		0	0	10	10
65	Prochlorperazine		100	100	91	79
66	Progesterone		0	0	0	0
67	Propionylpromazine		100	100	100	99
68	Quinacrine (Mepacrine)		85	98	100	100
69	Quinine		0	9	0	0
70	Ranitidien		100	99	61	47
71	Rosuvastatin		21	0	0	0

72	Sertraline		34	34	0	0
73	Silibinin		100	87	88	ND
74	Sulfasalazine		0	0	65	48
75	Sulindac		42	ND	72	63
76	Tamoxifen		59	83	91	91
77	Telmisartan		0	49	0	0
78	Thioridazine		100	100	100	100
79	Tolnaftate		71	36	0	26
80	Trifluoperazine		100	ND	100	100
81	Verapamil		85	71	100	100

82	Warfarin		69	80	100	96
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