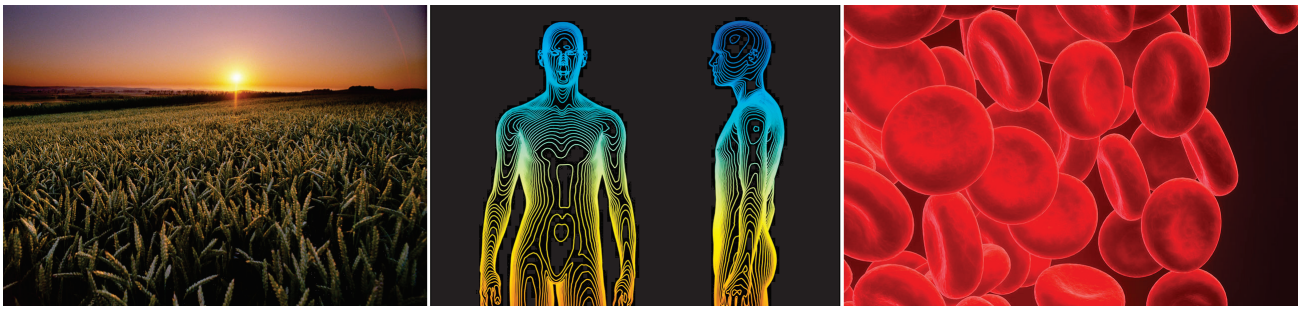

How Comprehensive is Your Analysis? Human Molecular Profiling Using High Resolution Time-of-Flight Mass Spectrometry

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Key Words: Metabolomics, Gas Chromatography, High Resolution Time-of-Flight Mass Spectrometry (GC-HRT)

1. Introduction

Since ancient times, urine has been used to diagnose human disease.[1,2] It was considered to be a divine fluid and window to the body. In fact, urine analysis was the primary diagnostic tool (uroscopy) from Babylonian to Victorian times. During the middle ages, physicians utilized a Matula for the collection of urine and a urine chart to evaluate a patient's health often without visitation. Urinalysis continues to be a very important part of modern laboratory medicine because urine is relatively free of interfering proteins and lipids, and large volumes are easily collected and stored. Furthermore, compounds such as drugs and metabolites in urine can be detected over extended periods of time.[3] In this study, urine was analyzed using high resolution time-of-flight mass spectrometry (HRT). The Pegasus® GC-HRT instrument was used to monitor behavior (e.g., drug abuse) and also to perform more comprehensive molecular profiling following proper urine sample preparation. The instrument's acquisition speed, mass accuracy (<1 ppm), and high resolving power (up to 50k) provides high quality spectral data that can be searched against large, well-established libraries. Confident compound identifications can be made rapidly through spectral comparisons and molecular, fragment, and adduct formula determinations using rich accurate mass data (Figure 1).

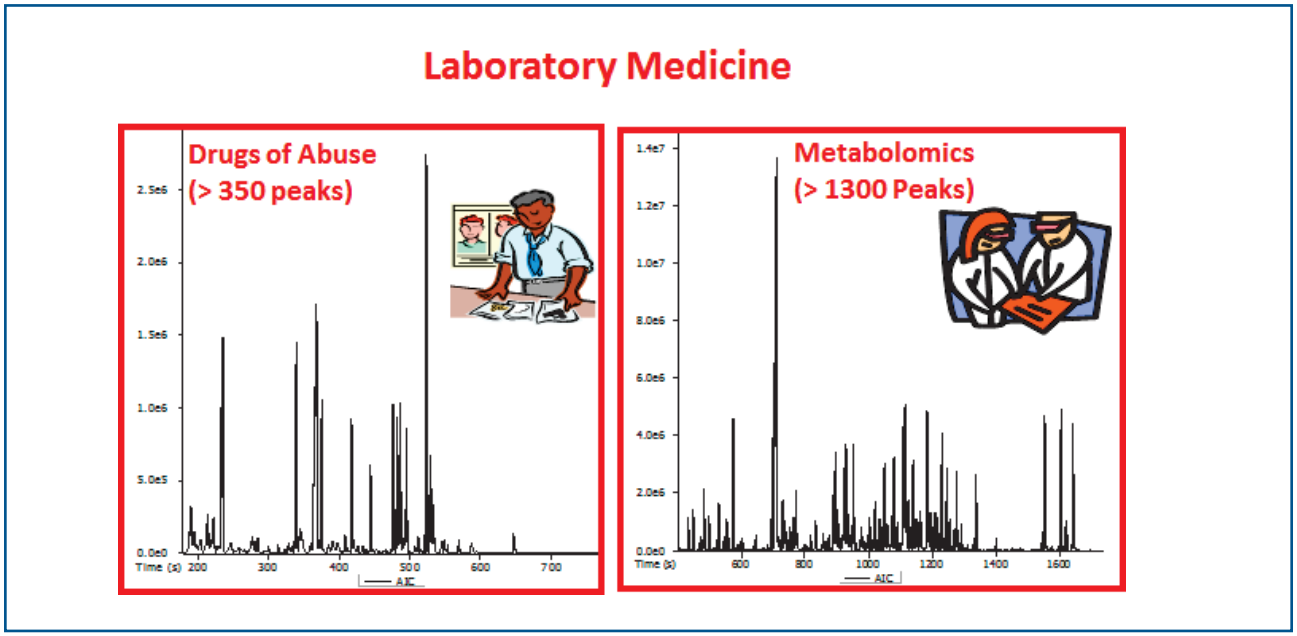

Figure 1. Laboratory medicine.

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2. Experimental

Sample preparation is a key part of the analysis workflow. Urine samples were prepared for either 1) drug monitoring or 2) detailed molecular profiling (derivatized) using different methods:

Method 1: 1.5 mL aliquots of urine were incubated with β-glucuronidase at 56°C for 1hr., transferred to HyperSep Verify-CX cartridges, extracted with 3% NH_3/CH_3OH (2 x 0.5 mL), and placed in 2mL GC vials for analysis.

Method 2: Urine (1mL) was treated with urease and then concentrated using a Speed Vac and lyophilizer. The residue was then derivatized using a two-step procedure: 1) Methoximation with 20 µL of MEOX (20 mg/mL methoxylamine hydrochloride in pyridine, 60 minutes at 60°C) followed by 2) treatment with 80 µL of MSTFA (60 minutes at 60°C). The corresponding GC-HRT instrument parameters used for sample analyses are shown in Tables 1 and 2.

Table 1. GC-High Resolution TOFMS (Pegasus GC-HRT) Conditions – Drug Monitoring.

Gas Chromatograph	**Agilent 7890 with 7693 Autosampler**
Injection	2µL, Splitless @ 280°C
Carrier Gas	He @ 1.0 ml/min, Constant Flow
Column	J&W VF-DA, 12 m x 0.20 mm i.d. x 0.33 µm (Agilent)
Temperature Program	70 °C (1 min), to 320 °C @ 25 °C/min (5 min)
Mass Spectrometer	**LECO Pegasus GC-HRT**
Transfer Line	300°C
Ion Source Temperature	250°C (EI); 200°C (CI)
Acquisition Mode	High Resolution, R = 25,000 (FWHM)
Ionization Mode	EI and CI (Reagent Gas: 5% NH_3 in CH_4)
Mass Range (m/z)	45-520 (EI); 60-800 (CI)
Acquisition Rate	10 spectra/s

Table 2. GC-High Resolution TOFMS (Pegasus GC-HRT) Conditions—Molecular Profiling.

Gas Chromatograph	**Agilent 7890 with 7693 Autosampler**
Injection	1µL, Splitless @ 250°C; 2µL for CI
Carrier Gas	He @ 1.0 ml/min, Constant Flow
Column	Rxi-5 Sil MS, 30 m x 0.25 mm i.d. x 0.25 µm (Restek, Bellefonte, PA, USA)
Temperature Program	70 °C (4 min), to 300 °C @ 10 °C/min (6 min)
Mass Spectrometer	**LECO Pegasus GC-HRT**
Transfer Line	300°C
Ion Source Temperature	250°C (EI); 200°C (CI)
Acquisition Mode	High Resolution, R = 25,000 (FWHM)
Ionization Mode	EI and CI (5% NH_3 in CH_4)
Mass Range (m/z)	45-520 (EI); 60-1000 (CI)
Acquisition Rate	10 spectra/s

3. Results and Discussion

A) Drug Monitoring: Comprehensive GC-HRT data acquisition was used to analyze urine samples. An example of the rich data obtained using this technology is displayed in the analytical ion chromatogram (AIC) below (Figure 2). A wide variety of compounds including nicotine, nicotine metabolites, and sterols were found in this sample. A representative set of compounds (1-34), their formulas, retention times, spectral similarities, and mass accuracy values (Ave. |ppm| =0.52) are listed in Table 3.

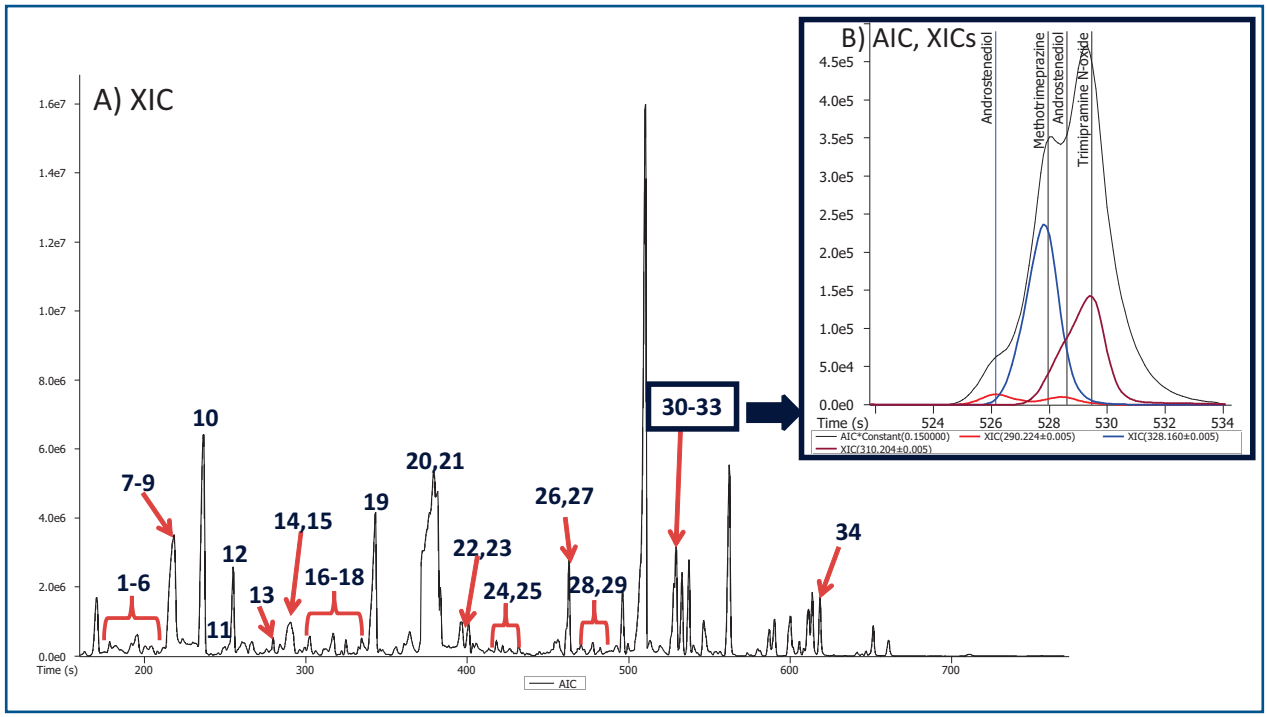

Figure 2. AIC of an underivatized urine sample prepared for a drug monitoring workflow.

Table 3. Representative Compounds in Urine.

Peak #	Name	Formula	R.T. (s)	Area	Similarity	PPM
1	3-Pyridinol	C_5H_5NO	183	6185856	812	-0.09
2	Dihydrothymine	$C_5H_8N_2O_2$	187	621464	746	0.73
3	N,2-Dimethylimidazole	$C_5H_8N_2$	188	178643	813	-0.47
4	Ethanol, 2-phenoxy-	$C_8H_{10}O_2$	194	4463665	809	-0.68
5	Octanoic acid, 2-methyl-	$C_9H_{18}O_2$	204	3662983	726	-0.29
6	Dihydrobenzofuran	C_8H_8O	208	399549	737	0.00
7	Creatinine ME	$C_5H_9N_3O$	218	36375929	773	-0.92
8	o-Ethynylaniline	C_8H_7N	221	3330816	792	-0.19
9	5-methylhydantoin	$C_4H_6N_2O_2$	232	24651088	793	-0.46
10	Nicotin	$C_{10}H_{14}N_2$	237	43628594	889	-1.04
11	1H-Indole, 3-methyl-	C_9H_9N	249	1481839	808	-1.11
12	2-(Methylmercapto)benzonitrile	C_8H_7NS	255	5421426	868	-0.74
13	Creatinine, 1-acetyl-	$C_6H_9N_3O_2$	276	1017562	767	-0.13
14	Methyl 4-hydroxyphenylacetate	$C_9H_{10}O_3$	288	1417270	738	-0.68
15	2,3,5-Trimethyl-6-ethylpyrazine	$C_9H_{14}N_2$	292	207732	734	-0.91
16	4 - vinyl - syringol	$C_{10}H_{12}O_3$	298	219900	764	0.44
17	Hexahydropyrrolizin-3-one	$C_7H_{11}NO$	302	2541535	730	-0.10

Peak #	Name	Formula	R.T. (s)	Area	Similarity	PPM
18	4-(3-Pyridyl-tetrahydrofuran-2-one	$C_9H_9NO_2$	317	1453261	862	-0.95
19	Cotinine	$C_{10}H_{12}N_2O$	343	23921315	865	-0.91
20	methyl 1H-indole-3-acetate	$C_{11}H_{11}NO_2$	371	1792478	792	-0.85
21	Hydroxycotinine	$C_{10}H_{12}N_2O_2$	382	63335156	795	-0.57
22	Theobromine	$C_7H_8N_4O_2$	397	5447784	790	-0.31
23	Proline anhydride	$C_{10}H_{14}N_2O_2$	401	5113606	854	-0.52
24	Acridine, 9-methyl-	$C_{14}H_{11}N$	410	519170	707	-0.47
25	Imidazo[2,1-a]isoquinoline	$C_{11}H_8N_2$	419	1196366	741	-0.13
26	Hydroxyandrostene	$C_{19}H_{30}O$	461	1102975	824	-0.81
27	Trimepramine	$C_{20}H_{26}N_2$	463	8095369	783	-0.47
28	Androsta-5,16-dien-3-ol	$C_{19}H_{28}O$	478	541953	813	0.22
29	Cyclo (Phe-Pro)	$C_{14}H_{16}N_2O_2$	492	1442590	747	-1.17
30	Androsterone	$C_{19}H_{30}O_2$	526	252420	834	0.06
31	LEVOMEPROPAZINE	$C_{19}H_{24}N_2OS$	528	12302443	883	0.13
32	Androstenediol	$C_{19}H_{30}O_2$	529	798453	736	0.06
33	Trimipramine-M (OH)	$C_{20}H_{26}N_2O$	530	9437706	903	-0.63
34	Cholesterol	$C_{27}H_{46}O$	619	799390	909	-0.35

This urine sample also contained over the counter drugs, pharmaceuticals, and their metabolites (Figure 3, Table 4). Mass accuracy values for these compounds ranged from -1.33 to 0.95 ppm with an average absolute value of 0.54 ppm.

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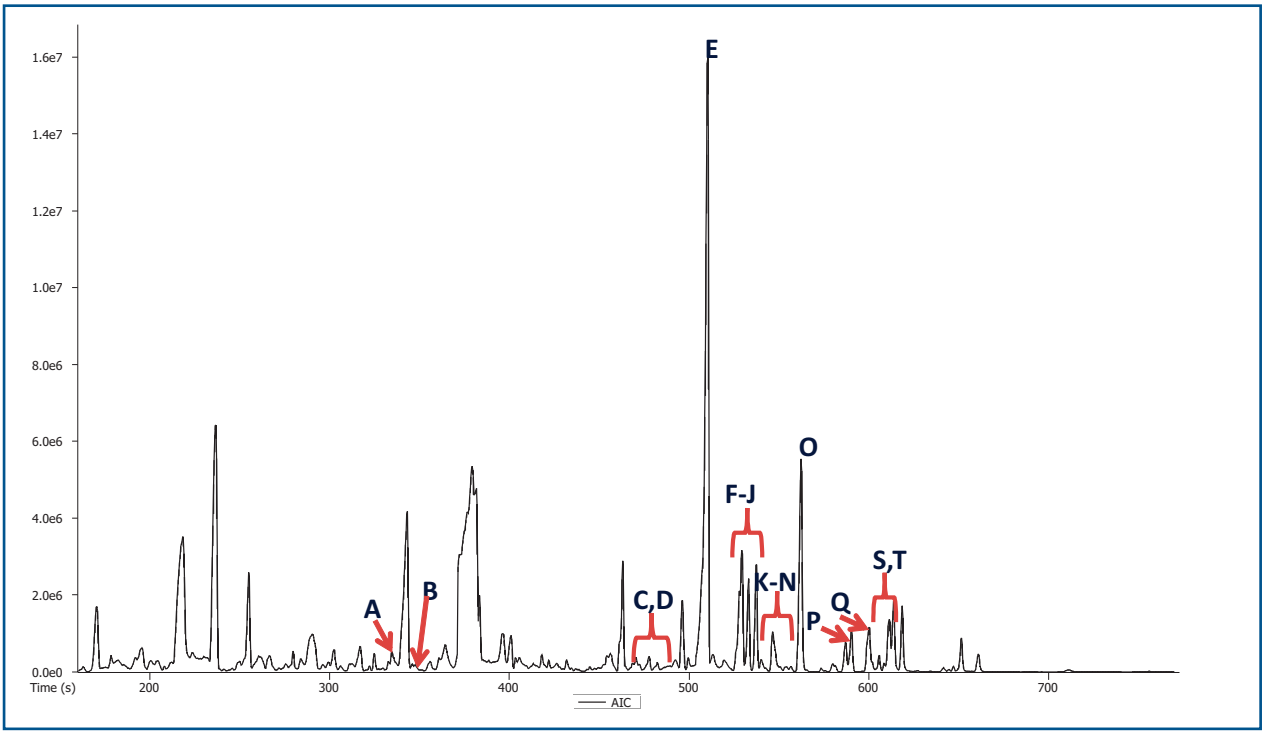

Figure 3. Over the Counter Drugs, Pharmaceuticals, and Metabolites in Urine Sample.

Table 4. Representative list of pharmaceuticals identified in urine sample.

Peak	*Name*	*Formula*	*R.T. (s)*	*Area*	*Similarity*	*Ion*	*PPM*
A	Ibuprofen	$C_{13}H_{18}O_2$	326.6	351091	745	$M^{\bullet+}$	-0.82
B	Tramadol-M	$C_{12}H_{14}O$	345.4	121179	669	$M^{\bullet+}$	-0.68
C	Chlorprothixene-M/A	$C_{13}H_7ClOS$	476.3	370789	729	$M^{\bullet+}$	-0.33
D	Chlorprothixene-M (-(CH_3)2N,-2H)	$C_{16}H_{11}ClS$	485.8	313724	761	$M^{\bullet+}$	-0.42
E	Citalopram	$C_{20}H_{21}FN_2O$	510.5	72567737	873	$M^{\bullet+}$	0.33
F	Levomepromazine	$C_{19}H_{24}N_2OS$	528	12302443	883	$M^{\bullet+}$	0.13
G	Trimipramine-M (OH)	$C_{20}H_{26}N_2O$	529.5	9437706	903	$M^{\bullet+}$	-0.63
H	Quetiapine-M/A (N-Desalkyl,desulfo) ME	$C_{18}H_{19}N_3$	533.1	7754648	897	$M^{\bullet+}$	-0.39
I	Quetiapine-M (Desalkyl) ME	$C_{18}H_{19}N_3S$	537.4	4936057	842	$M^{\bullet+}$	-0.28
J	Trimipramine-M (OH,OCH_3)	$C_{21}H_{28}N_2O_2$	538.8	263624	683	$M^{\bullet+}$	0.56
K	Quetiapine-M/A (N-Desalkyl,desulfo)	$C_{17}H_{17}N_3$	540.2	1287954	844	$M^{\bullet+}$	-0.04
L	Quetiapine-M (Desalkyl)	$C_{17}H_{17}N_3S$	546.4	4157125	900	$M^{\bullet+}$	-0.75
M	3-hydroxytrimeprazine	$C_{18}H_{22}N_2OS$	547.7	2440199	711	$M^{\bullet+}$	-0.15
N	Quetiapine-M (N-Ethyl,desulfo)	$C_{19}H_{21}N_3$	549.3	231108	792	$M^{\bullet+}$	0.89
O	Hydroxylevomepromazine Isomer	$C_{19}H_{24}N_2O_2S$	562.4	30115502	742	$M^{\bullet+}$	0.31
P	7-Hydroxylevomepromazine	$C_{19}H_{24}N_2O_2S$	589.1	1001851	792	$M^{\bullet+}$	0.95
Q	Levomepromazine-M/A (sulfoxide)	$C_{19}H_{24}N_2O_2S$	600.2	5887408	769	$M^{\bullet+}$	0.28
R	Quetiapine-M (Formyloxy)	$C_{20}H_{21}N_3O_2S$	610.8	1447470	859	$M^{\bullet+}$	-0.38
S	Quetiapine-M (-$CH_2OC_2H_4OH$,Oxo)	$C_{18}H_{17}N_3OS$	613.8	2986947	761	$M^{\bullet+}$	-1.08
T	Quetiapine	$C_{21}H_{25}N_3O_2S$	651.4	1805190	886	$M^{\bullet+}$	-1.33

The mass spectra for citalopram, an anti-depressant, is shown in Figure 4. Mass accuracy and library match values for citalopram were 0.33 ppm and 873/1000, respectively. Complementary CI-HRT data was particularly useful for identification purposes when the molecular ion was either absent or very small, such as in the case of quetiapine (Figure 5). A strong protonated molecular ion was clearly visible in the CI-HRT spectrum of this anti-psychotic at m/z = 384.17450 (1.25 ppm).

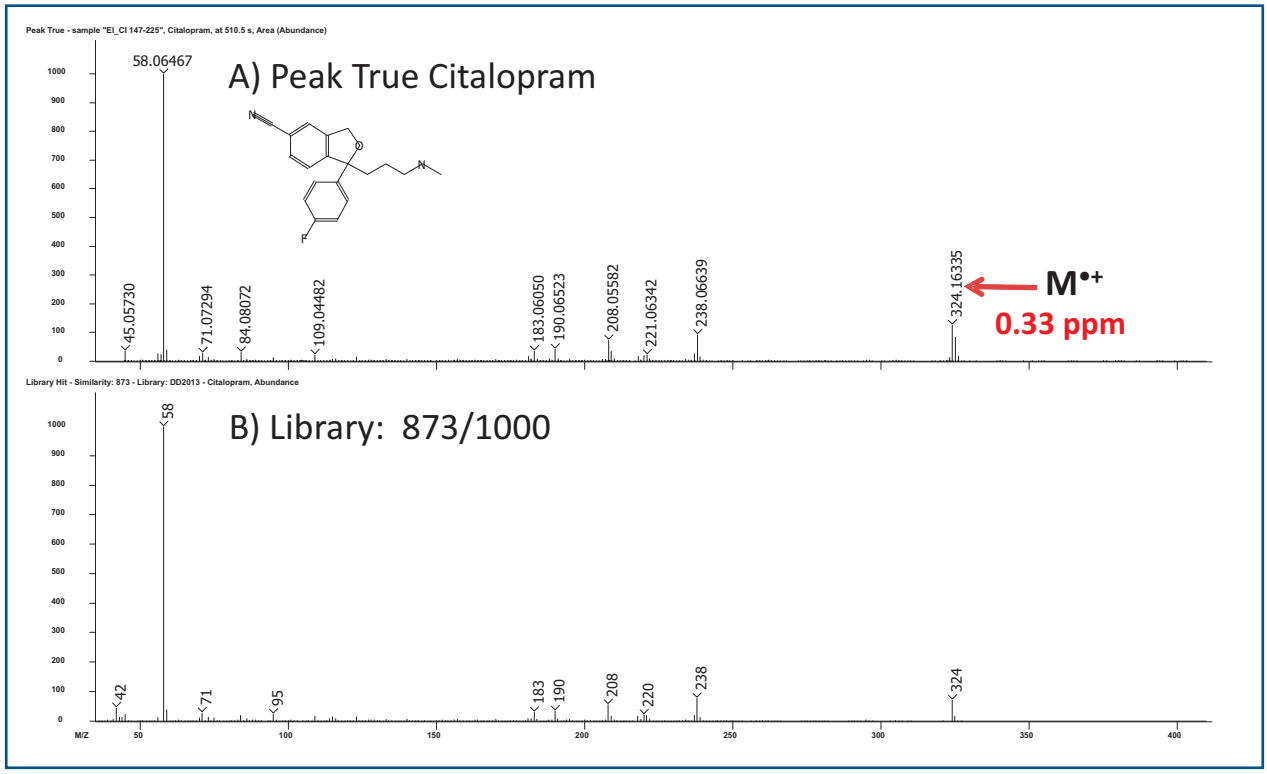

Figure 4. A) Peak True and B) Library Spectra for Citalopram.

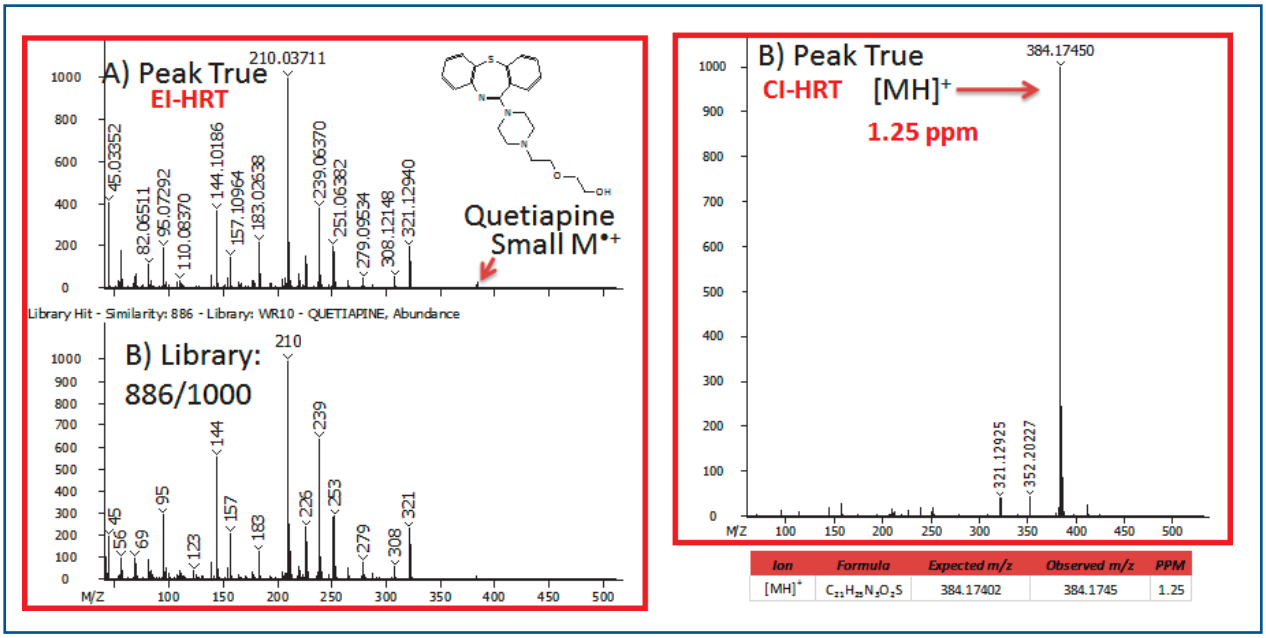

Figure 5. A) Peak True (EI-HRT), B) Library (EI-MS), and C) Peak True (CI-HRT) Spectra for Quetiapine.

B) Comprehensive Molecular Profiling: A more detailed profile of an individual can be obtained through derivatization of urine using the two-step process described in the experimental section (Figure 6). Derivatization greatly expands analysis coverage (>1300 peaks) to include ionic and polar compounds such as acids, diacids, amino acids, monosaccharides, fatty acids, disaccharides, etc. (Figure 7). Table 5 shows a small representative list of these compounds with an average spectral similarity of 851/1000. Confident and rapid identification of metabolites is accomplished through spectral similarity searches and leveraging accurate mass ions for formula determinations of ions in the EI-HRT spectra (Figure 8). Accurate mass ions for the quinic acid fragments $[M\text{-}C_7H_{19}O_3Si_2]^+$ and $[M\text{-}C_4H_9O_2Si]^+$ were associated with mass accuracies of 0.56 and -0.65 ppm, respectively. In addition, molecular adduct ions in the complementary CI-HRT data can be utilized to confirm assignments as shown in the corresponding CI-HRT of this acid. The protonated molecular ion, $[MH]^+$ at m/z =553.26832 displayed a mass accuracy of 0.04 ppm.

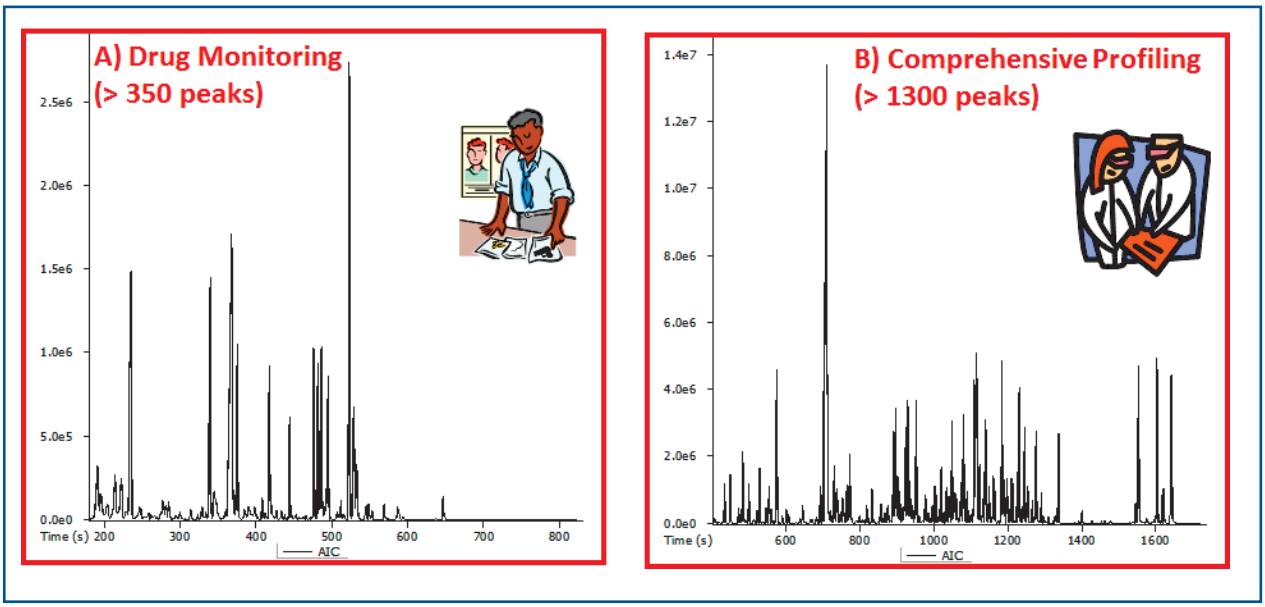

Figure 6. A) Drug monitoring and B) Comprehensive profiling of a urine sample utilizing differing sample preparation approaches.

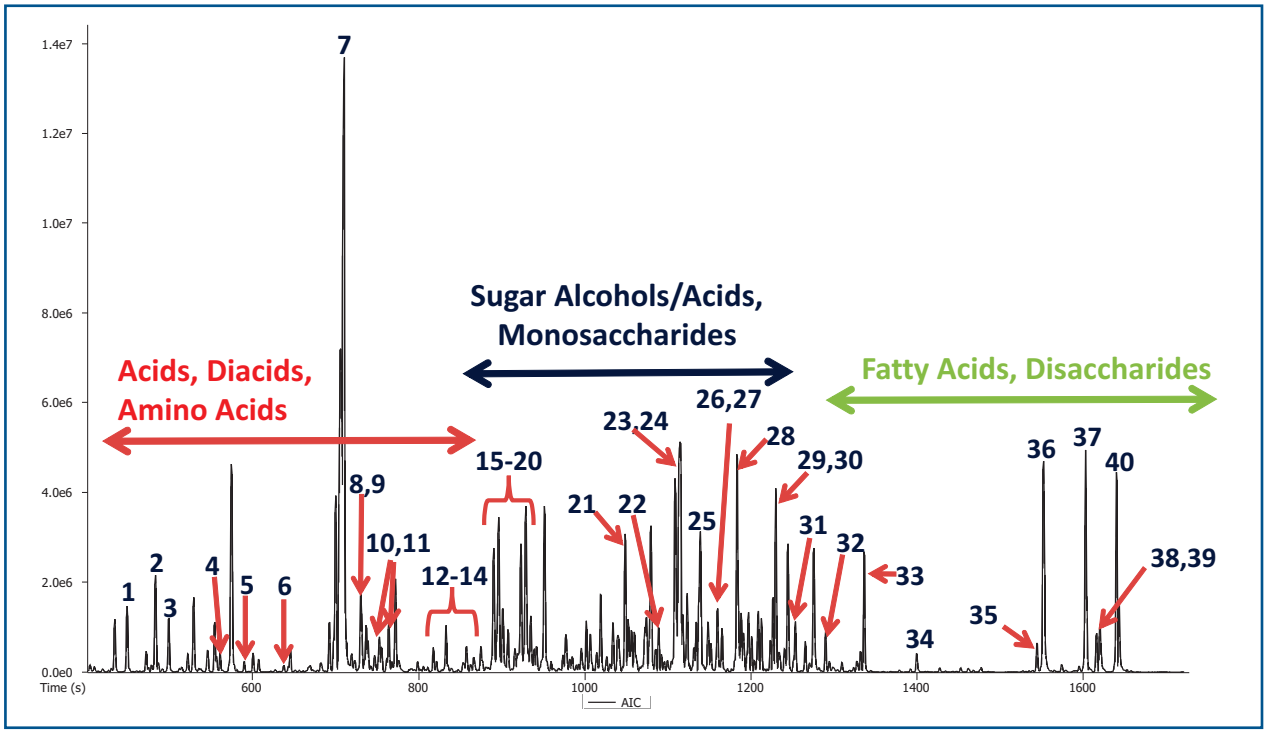

Figure 7. An AIC of a Derivatized Urine Sample.

Table 5. Representative Compounds in Derivatized Urine.

Peak	Name	Formula	R.T. (s)	Area	Similarity
1	3-Hydroxypyridine TMS	$C_8H_{13}NOSi$	449	15623393	781
2	Lactic acid 2TMS	$C_9H_{22}O_3Si_2$	483	9907588	925
3	Glycolic acid 2TMS	$C_8H_{20}O_3Si_2$	499	6617490	940
4	Oxalic acid 2TMS	$C_8H_{18}O_4Si_2$	561	2282579	784
5	3-Hydroxyisobutyric acid 2TMS	$C_{10}H_{24}O_3Si_2$	590	1340172	865
6	3-Hydroxyvaleric acid 2TMS	$C_{11}H_{26}O_3Si_2$	638	592061	881
7	Phosphoric acid 3TMS	$C_9H_{27}O_4PSi_3$	710	251511040	802
8	Glycine 3TMS	$C_{11}H_{29}NO_2Si_3$	730	9514329	889
9	Methylmalonic acid 2TMS	$C_{10}H_{22}O_4Si_2$	731	9167327	868
10	Glyceric acid 3TMS	$C_{12}H_{30}O_4Si_3$	751	800170	857
11	Uracil 2TMS	$C_{10}H_{20}N_2O_2Si_2$	755	1376869	897
12	3-Deoxytetronic acid 3TMS	$C_{13}H_{32}O_4Si_3$	818	1975789	828
13	3,4-Dihydroxybutanoic acid 3TMS	$C_{13}H_{32}O_4Si_3$	833	5525926	917
14	2-Methylmalic acid 3TMS	$C_{14}H_{32}O_5Si_3$	866	1962484	787
15	Threitol 4TMS	$C_{16}H_{42}O_4Si_4$	890	9912288	916
16	Erythritol 4TMS	$C_{16}H_{42}O_4Si_4$	896	11656512	928
17	5-oxo-Proline 2TMS	$C_{11}H_{23}NO_3Si_2$	901	11765910	910
18	2-Hydroxyglutaric acid 3TMS	$C_{14}H_{32}O_5Si_3$	907	1523178	734
19	Creatinine 3TMS	$C_{13}H_{31}N_3OSi_3$	929	18522090	901
20	Threonic acid 4TMS	$C_{16}H_{40}O_5Si4$	935	4557440	924

Peak	Name	Formula	R.T. (s)	Area	Similarity
21	Arabitol 5TMS	$C_{21}H_{54}O_5Si_5$	1049	9711476	933
22	Ribonic acid 5TMS	$C_{20}H_{50}O_6Si_5$	1089	3706793	869
23	Hippuric acid TMS	$C_{12}H_{17}NO_3Si$	1115	51167658	780
24	Ribofuranose 4TMS	$C_{17}H_{42}O_5Si_4$	1118	6671597	751
25	Quinic acid 5TMS	$C_{22}H_{52}O_6Si_5$	1139	12127180	845
26	Altronic acid, 1,4-lactone 4TMS	$C_{18}H_{42}O_6Si_4$	1160	2199226	615
27	Quinic acid-Isomer 5TMS	$C_{22}H_{52}O_6Si_5$	1172	364426	680
28	Mannitol 6TMS	$C_{24}H_{62}O_6Si_6$	1183	18592900	909
29	Gluconic acid, (6TMS)	$C_{24}H_{60}O_7Si_6$	1227	6344075	855
30	Palmitic acid TMS	$C_{19}H_{40}O_2Si$	1230	10291823	912
31	Salicyluric acid 2TMS	$C_{15}H_{25}NO_4Si_2$	1252	798597	703
32	3-Hydroxyhippuric acid 2TMS	$C_{15}H_{25}NO_4Si_2$	1290	2592321	845
33	Stearic acid TMS	$C_{21}H_{44}O_2Si$	1337	5947507	928
34	Pseudourindine 5TMS	$C_{24}H_{52}N_2O_6Si_5$	1400	2121203	865
35	Lactulose 8TMS	$C_{36}H_{86}O_{11}Si_8$	1545	2385317	857
36	Lactose 8TMS, Isomer 1	$C_{36}H_{86}O_{11}Si_8$	1552	34444484	930
37	Lactose 8TMS, Isomer 2	$C_{36}H_{86}O_{11}Si_8$	1603	19257409	920
38	Lactose MEOX, 8TMS	$C_{37}H_{89}NO_{11}Si_8$	1616	2647365	832
39	Maltose MEOX, 8TMS, Isomer 1	$C_{37}H_{89}NO_{11}Si_8$	1620	4930508	813
40	Maltose MEOX, 8TMS, Isomer 2	$C_{37}H_{89}NO_{11}Si_8$	1640	10556846	856

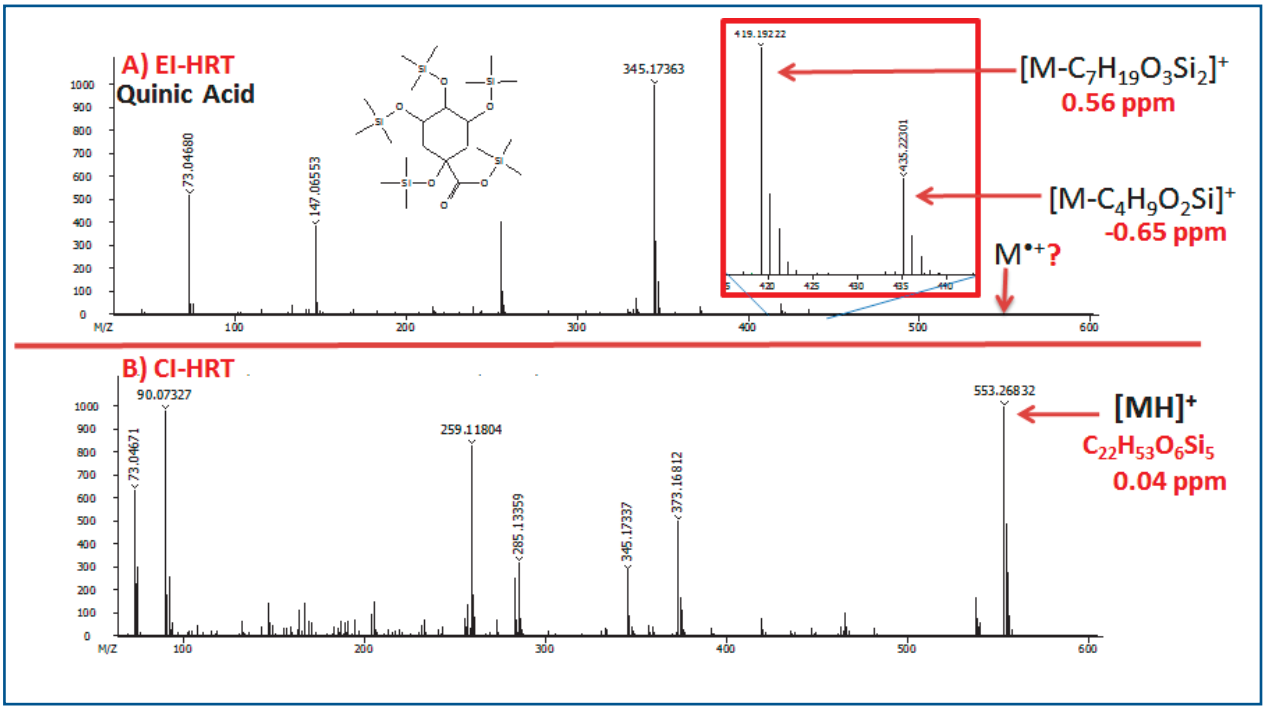

Figure 8. A) EI-HRT and B) CI-HRT Spectra for Quinic Acid.

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4. Conclusion

The LECO Pegasus GC-HRT is an excellent tool for drug monitoring and molecular profiling using urine samples. EI and complementary CI data facilitated compound identification through spectral similarity searches of large databases and robust formula determinations for fragment, molecular, and adduct ions. More detailed patient profiles were obtained with comprehensive sample preparation and GC-HRT data acquisition.

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