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ORIGINAL ARTICLE

Construction of calibration-locking databases for rapid and reliable drug screening by gas chromatography-mass spectrometry

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Abstract Unique calibration-locking databases were constructed for rapid and semiquantitative drug screening by gas chromatography-mass spectrometry (GC-MS). In addition to the free-drug database of 127 drugs, a drug database with acetylating reagents was constructed to increase the number of detectable compounds in the analysis by GC-MS; 156 drugs, including 30 drugs of abuse, 42 hypnotics and their metabolites, 18 antipsychotic drugs, 15 antidepressants, and 12 antipyretic analgesic agents, were registered with parameters, such as the mass spectrum, retention time, qualifier ion/target ion percentage, and calibration curve using the novel GC-MS software NAGINATA. Diazepam-d_5 was used as internal standard for construction of each calibration curve in the range of 0.01–5.0 μg/ml for most drugs. We examined the applicability of the constructed database to analyzing whole blood samples spiked with 40 drugs most commonly encountered in toxicological cases in Japan. The drugs in blood were extracted using enhanced polymer columns (Focus), subjected to GC-MS after incubation with acetylating reagents, and screened by the drug database. Among the 40 drugs examined, 38 and 30 drugs were successfully identified at the level of 1 and 0.1 μg/ml, respectively, without using standard compounds. The time required for data analysis was less than 1 min, and semiquantitative data were also obtained simultaneously. Because new drugs and metabolites can easily be added to the databases, we can recommend them as useful tools in clinical and forensic toxicological screening.

Keywords Calibration-locking database · Systematic toxicological analysis · Enhanced polymer column · GC-MS · Acetylation · NAGINATA

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Introduction

Analysis of drugs and/or poisons in biological samples is an important routine task in clinical and forensic toxicology. Simple and rapid systematic toxicological analysis (STA), which can cover hundreds of relevant drugs, poisons, and/or their metabolites, is most desirable for such purpose. Many chromatographic techniques have been used for STA, such as gas chromatography (GC) with different detectors, GC coupled with mass spectrometry (GC-MS), liquid chromatography (LC) with diode-array detection, LC with single-stage mass spectrometry (LC-MS), LC-MS-MS, or LC-time-of-flight MS (LC-TOFMS) [1–6]. Of these methods, LC-TOFMS is considered to be the most powerful tool, because this technique may detect most of the drugs/poisons and their metabolites without using standard compounds [4]. However, this approach is limited by the high cost of the instrumentation. A dual-column GC with two nitrogen/phosphorus detectors has been used for quantitative screening of basic drugs in whole blood [7]. The system is simple and economical, and also gives quantitative information, but requires all reference standards to set up the system in each laboratory. The mass spectral libraries obtained by LC-MS or LC-MS-MS systems can

be used only in each instrument due to the difference in mass spectra from individual instruments [5]. Therefore, GC-MS analysis using the commercially available mass spectral libraries, such as Wiley, NIST, and PMW_TOX is still the gold standard for STA in most small laboratories. However, this kind of approach requires considerable time and an experienced analyst for data analysis, because these libraries mainly give information only for mass spectra. Therefore, a rapid screening method that gives reliable qualitative and quantitative information without using standard compounds is needed.

New GC-MS software named "NAGINATA" was recently developed by Nishikawa Keisoku (Tokyo, Japan) on the basis of the concept of Kadokami et al. [8] with several modifications. This software is designed for GC-MS system quality control and data analysis support as an add-on software of Chemistation (Agilent, Santa Clara, CA, USA) to operate an Agilent 6890GC/5973 or 5975MSD instrument. A system performance check using a criterion sample mix solution can compensate for instrument-to-instrument and day-to-day condition variations before sample measurements. Automatic compound search based on the calibration-locking database including the retention time, calibration curve, and electron impact ionization (EI) mass spectrum of each compound can be performed after sample recording. Then, confirmation and tentative quantitation values of all compounds are quickly obtained as analytical results without preparing respective standard compounds. This software was originally on the market as a preset database for about 600 compounds, such as pesticides, for environmental analyses, but no application to toxicological screening had been carried out.

We, therefore, constructed a preliminary database for 30 drugs of abuse using this software, and examined the usefulness of this approach by analyzing urine samples in forensic autopsy cases [9]. In this study, we have extended this line of experiments to screening 156 drugs or their metabolites, which will prove very useful in forensic and clinical toxicology.

Materials and methods

Reagents

The drug standards were either provided from various pharmaceutical companies or purchased from Wako (Osaka, Japan), Cerilliant (Round Rock, TX, USA), and Sigma-Aldrich (St. Louis, MO, USA). Some drugs of abuse were synthesized in our laboratory or provided from the Ministry of Health, Labour, and Welfare, Japan. Diazepam-d_5 used as internal standard (IS) was purchased from Lipomed (Cambridge, MA, USA); trifluoroacetic acid (TFA) and ethyl acetate from Wako; acetic anhydride from Sigma-Aldrich; pyridine (silylation grade) from Pierce of ThermoFisher Scientific (Milwaukee, WI, USA); Focus columns from Varian (Lake Forest, CA, USA). Other chemicals were of analytical reagent grade.

Standard solutions

Most drugs (5 mg in a free form) were dissolved in methanol and the volume was adjusted to 5 ml to obtain a concentration of 1000 ng/μl. These solutions were further diluted in methanol to 100 and 10 ng/μl.

Biological samples

Drug-free human whole blood was obtained from healthy volunteers and used as control samples. Whole blood and urine samples obtained at autopsies were stored at –20°C until analysis.

Construction of calibration curves

A total of 210 drugs, including 33 drugs of abuse, 54 hypnotics and their metabolites, 27 antipsychotic drugs, 17 antidepressants, and 19 antipyretic analgesic agents, were tested with and without acetylating reagents at the level of 1 μg/ml (20 ng on column); 127 and 156 drugs listed in Table 1 were selected for constructing drug databases with and without acetylating reagents, respectively.

Known amounts of 127 drugs (0.01, 0.05, 0.1, 0.5, 1, or 5 μg) were dissolved in 100 μl ethyl acetate containing 1 μg of IS, and a 2-μl aliquot of each solution was subjected to GC-MS, with which the performance of the tool "System Performance Check" in NAGINATA software had been evaluated. The calibration curves for the "free-drug database" were thus constructed. The same amounts of 156 drugs (0.01, 0.05, 0.1, 0.5, 1, or 5 μg) were mixed with 50 μl each of pyridine and acetic anhydride. The mixture was incubated at 60°C for 30 min, and was evaporated to dryness at room temperature under a stream of nitrogen. The residue was dissolved in 100 μl of ethyl acetate containing 1 μg of IS. A 2-μl aliquot of the solution was subjected to GC-MS to construct calibration curves for the "drug database with acetylating reagents."

GC-MS conditions

The apparatus used was an Agilent 6890 GC combined with an Agilent 5973 MS. NAGINATA software was

Table 1 Drug parameters for the two databases

Compound	Free-drug database				Drug database with acetylating reagents			
	Retention time (min)	Target ion (*m/z*)	Qualifier ion (*m/z*)	Calibration range (μg/ml)	Retention time (min)	Target ion (*m/z*)	Qualifier ion (*m/z*)	Calibration range (μg/ml)
Bromisovalum	6.099	137	139	0.05–5	6.125	137	139	0.1–5
Allylisopropylacetylurea	6.337	98	126	0.05–5	6.365	98	126	0.05–5
Dimethylamphetamine	6.682	72	91	0.05–5	6.717	72	91	0.01–5
Nicotine	7.471	84	133	0.05–5	7.505	84	133	0.01–5
Propofol	7.522	163	178	0.5–5	—			
Metharbital	8.23	155	170	0.01–5	8.269	155	170	0.1–5
Acetylpheneturide	8.499[b]	91	135	0.01–5	8.522[b]	91	135	0.01–1
Methylephedrine	7.974	72	77	0.1–5	8.571[a]	72	105	0.01–5
Barbital	8.584	156	141	0.01–5	8.594	156	141	0.01–5
β-Phenethylamine	—				8.643[a]	104	163	0.1–5
Amphetamine	5.627	91	65		8.697[a]	86	118	0.1–5
Methamphetamine	6.145	58	91	0.1–5	9.255[a]	58	100	0.01–5
Ethenzamide	9.259	120	92	0.01–5	9.279	120	92	0.05–5
Ibuprofen	—				9.306	161	206	0.5–5
Phenacetin	9.85	108	179	0.01–5	9.864	108	179	0.01–5
Cotinine	9.92	98	176	0.01–5	9.939	98	176	0.05–5
Amobarbital	10.002	156	141	0.01–5	10.012	156	141	0.01–5
Phenylpropanolamine	7.322	77	51	0.5–5	10.165[a]	86	107	0.01–5
Pentobarbital	10.151	156	141	0.05–5	10.168	156	141	0.01–5
p-Methoxyamphetamine	7.711	122	78	0.1–5	10.265[a]	148	121	0.05–5
Mexiletine	7.825	58	77	0.01–5	10.345[a]	100	58	0.01–5
Acetaminophen	9.77	109	151	0.5–5	10.351[a]	109	151	0.01–5
Secobarbital	10.458	168	195	0.01–5	10.471	168	195	0.01–5
Ephedrine	7.664	58	77	0.1–5	10.579[a]	58	100	0.01–5
Caffeine	10.74	194	109	0.01–5	10.757	194	109	0.01–5
p-Methoxymethamphetamine	9.938	58	149	0.05–5	10.774[a]	58	148	0.01–5
MDA	8.439	136	77	0.1–5	10.871[a]	162	135	0.01–5
Glutethimide	10.883	189	117	0.01–5	10.9	189	117	0.01–5
Thiopental	10.906	172	157	0.1–5	10.926	172	157	0.1–5
Diphenhydramine	10.944	58	165	0.1–5	10.962	58	165	0.05–5
Antipyrine	10.986	188	77	0.05–5	11.001	188	96	0.01–5
4-Hydroxyamphetamine	—				11.031[a]	134	176	0.01–5
Lidocaine	11.025	86	58	0.01–5	11.042	86	58	0.01–5
Mephobarbital	11,154	218	117	0.01–5	11.17	218	117	0.1–5
Thiamylal	11.174	184	169	0.1–5	11.191	184	169	0.5–5
1-Benzylpiperazine	8.522	91	134	0.1–5	11.329[a]	91	146	0.01–5
4-Methylthioamphetamine	8.996	138	122	0.1–5	11.34[a]	164	137	0.01–5
MDMA	8.791	58	135	0.05–5	11.359[a]	58	162	0.01–5
1-(3-Trifluoromethylphenyl)piperazine	8.768	188	230	0.05–5	11.365[a]	200	272	0.1–5
Isopropylantipyrine	11.38	215	230	0.01–5	11.397	215	230	0.01–5
4-Hydroxymethamphetamine	—				11.494[a]	58	100	0.01–5
Phenobarbital	11.483	204	232	0.5–5	11.497	204	232	0.5–5

Table 1 *Continued*

Compound	Free-drug database				Drug database with acetylating reagents			
	Retention time (min)	Target ion (*m/z*)	Qualifier ion (*m/z*)	Calibration range (μg/ml)	Retention time (min)	Target ion (*m/z*)	Qualifier ion (*m/z*)	Calibration range (μg/ml)
Cyclobarbital	11.539	207	141	0.1–5	11.557	207	141	0.01–5
Baclofen	—				11.598[c]	138	237	0.01–5
Dantrolene	—				11.66[a]	214	184	0.1–5
MBDB	9.399	72	135	0.05–5	11.686[a]	72	176	0.01–5
5-Methoxy-*N,N*-dimethyltryptamine	11.694	58	218	0.05–5	11.711	58	218	0.05–5
Chlorpheniramine	11.734	203	58	0.1–5	11.751	203	58	0.01–5
Methylone	—				11.876[a]	58	100	0.01–5
Mescaline	9.871	182	167	0.5–5	12.015[a]	194	181	0.01–5
Mepivacaine	12.122	98	70	0.01–5	12.134	98	70	0.01–5
Methylphenidate	10.162	84	56	0.05–5	12.168[a]	84	126	0.01–5
Diphenylpyraline	12.26	99	167	0.05–5	12.277	99	167	0.01–5
Clofedanol	12.248	58	254	0.05–5	12.374[a]	58	296	0.1–5
Psilocin	—				12.408[a]	58	146	0.05–5
α-Methyltryptamine	10.242	131	130	0.5–5	12.439[a]	130	157	0.1–5
Proxyphylline	12.191	194	180	0.05–5	12.453[a]	220	280	0.01–5
5-Methoxy-*N,N*-methylisopropyltryptamine	12.471	86	160	0.05–5	12.488	86	160	0.05–5
Diclofenac	12.478[b]	214	277	0.01–5	12.5[b]	214	277	0.01–5
Dextromethorphan	12.46	59	271	0.1–5	12.503	59	271	0.01–5
Lorazepam	—				12.572[b]	253	287	0.01–5
2C-B	10.557	230	232	0.5–5	12.595[a]	242	148	0.05–5
1-(3-Chlorophenyl)piperazine	—				12.614[a]	166	238	0.01–5
1-(4-Methoxyphenyl)piperazine	10.219	150	192	0.1–5	12.638[a]	162	234	0.1–5
Bromocriptine	12.673	209	154		12.679	154	209	0.01–5
Ketamine	10.934	180	209	0.01–5	12.688[a]	216	208	0.01–5
Amitriptyline	12.728	58	202	0.1–5	12.741	58	202	0.01–5
Fluvoxamine	10.947	71	187	0.1–5	12.832[a]	86	187	0.05–5
Mianserin	12.821	193	264	0.05–5	12.839[a]	193	264	0.01–5
Trimipramine	12.843	58	249	0.05–5	12.854	58	249	0.01–5
Imipramine	12.866	234	58	0.05–5	12.879	58	234	0.01–5
Primidone	12.964	146	190	0.05–5	12.98	146	190	0.05–5
Medazepam	12.986	207	242	0.01–5	12.997	207	242	0.01–5
5-Methoxy-*N,N*-diisopropyltryptamine	13.014	114	160	0.05–5	13.025	114	160	0.01–5
Trihexyphenidyl	13.015	98	218	0.01–5	13.026	98	218	0.01–5
Milnacipran	—				13.038	204	288	0.01–5
Chlormezanone	13.043	98	152	0.05–5	13.057	98	152	0.1–5
4-Aminoantipyrine	11.637	84	203	0.5–5	13.072[a]	56	245	0.05–1
2C-I	—				13.104[a]	290	349	0.05–5
2-Oxoquazepam	13.105	342	370	0.05–5	13.117	342	370	0.01–5
5-Methoxy-*N,N*-dipropyltryptamine	13.105	114	160	0.05–5	13.121	114	160	0.01–5
4-Hydroxy-*N,N*-methylisopropyltryptamine	—				13.156	86	146	0.01–5
Bupivacaine	13.166	140	84	0.01–5	13.167	140	84	0.01–5
2C-T-2	—				13.174[a]	224	283	0.01–5

Table 1 *Continued*

Compound	Free-drug database				Drug database with acetylating reagents			
	Retention time (min)	Target ion (*m/z*)	Qualifier ion (*m/z*)	Calibration range (μg/ml)	Retention time (min)	Target ion (*m/z*)	Qualifier ion (*m/z*)	Calibration range (μg/ml)
Promethazine	13.166	72	180	0.1–5	13.18	72	180	0.05–5
Biperiden	13.17	98	218	0.05–5	13.184	98	218	0.01–5
Atropine	12.758	124	289	0.1–5	13.232[a]	124	94	0.01–5
Pilsicainide	13.248	110	272	0.05–5	13.26	110	272	0.05–5
Setiptiline	13.249	83	261	0.05–5	13.265	83	261	0.05–5
Pentazocine	13.106	217	202	0.1–5	13.374[a]	259	110	0.01–5
Carbamazepine	13.375	193	236	0.05–5	13.392	193	236	0.05–5
5-Methoxy-α-methyltryptamine	—				13.434[a]	160	187	0.1–5
Sulpyrine	—				13.443[c]	56	259	0.01–5
2C-T-7	—				13.528[a]	238	297	0.05–5
Melatonin	13.552	160	173	0.1–5	13.556	160	232	0.5–5
Dosulepin	13.648	58	202	0.05–5	13.66	58	202	0.01–5
Fludiazepam	13.688	274	301	0.01–5	13.695	274	301	0.01–5
Clomipramine	13.747	58	268	0.05–5	13.797	58	268	0.01–5
Diprophylline	—				13.836[a]	180	338	0.1–5
Diazepam-d_5 (internal standard)	13.917	261	287		13.923	261	287	
Diazepam	13.923	256	283	0.01–5	13.923	256	283	0.01–5
Quazepam	14.008	245	386	0.01–5	14.015	245	386	0.01–5
3-Hydroxy-2-oxoquazepam	—				14.089[a]	357	386	0.05–5
Dihydrocodeine	13.708	301	164	0.1–5	14.107[a]	343	284	0.01–5
Chlorpromazine	14.172	58	318	0.1–5	14.185	58	318	0.01–5
N-Desmethyldiazepam	14.228	242	269	0.01–5	14.22	242	269	0.1–5
Chlordiazepoxide	14.255[b]	282	220	0.01–5	14.254[b]	282	220	0.05–5
Codeine	13.7	299	162	0.1–5	14.266[a]	282	341	0.01–5
Clotiazepam	14.272	289	318	0.01–5	14.278	289	318	0.01–5
Levomepromazine	14.289	58	328	0.05–5	14.292	58	328	0.01–5
Flumazenil	14.338	229	257	0.01–5	14.347	229	303	0.01–5
Clobazam	14.483	255	300	0.01–5	14.492	255	300	0.01–5
Zotepine	14.546	58	72	0.05–5	14.557	58	72	0.01–5
Cloxazolam	14.595	305	262	0.5–5	—			
Midazolam	14.638	310	325	0.01–5	14.647	310	325	0.01–5
Desalkylflurazepam	13.974	259	287	0.05–5	13.976	259	288	0.5–5
Delorazepam	14.704	275	304	0.05–5	14.706	275	304	0.1–5
Flunitrazepam	14.753	285	312	0.01–5	14.758	285	312	0.05–5
Bromazepam	14.831	236	315	0.5–5	14.831	236	315	1–5
Morphine	—				14.866[a]	268	327	0.05–5
Nortriptyline	12.814	202	215	0.1–5	14.906[a]	232	217	0.01–5
Dibucaine	15.06	86	116	0.05–5	15.065	86	116	0.01–5
Desipramine	12.976	234	193	0.1–5	15.126[a]	208	308	0.01–5
Temazepam	—				15.144[a]	271	300	0.01–5
2-Hydroxyethylflurazepam	15.015	288	273	0.05–5	15.322[a]	87	314	0.01–5
Quinidine	15.771	136	324	0.5–5	15.489[a]	136	366	0.01–5

Table 1 *Continued*

Compound	Free-drug database				Drug database with acetylating reagents			
	Retention time (min)	Target ion (*m/z*)	Qualifier ion (*m/z*)	Calibration range (μg/ml)	Retention time (min)	Target ion (*m/z*)	Qualifier ion (*m/z*)	Calibration range (μg/ml)
Flurazepam	15.552	86	58	0.1–5	15.56	86	58	0.01–5
Olanzapine	15.336	242	229	0.5–5	15.613[a]	284	242	0.01–5
Maprotiline	13.431	277	203	0.1–5	15.695[a]	291	218	0.01–5
Lormetazepam	—				15.696[a]	305	291	0.01–5
Tiapride	15.762	86	213	0.1–5	15.766	86	213	0.1–5
Zolpidem	15.8	235	307	0.05–5	15.798	235	307	0.01–5
Bisacodyl	15.856	276	361	0.1–5	15.871	276	361	0.1–5
α-Hydroxymidazolam	—				16.095[a]	310	340	0.01–5
Estazolam	16.548	259	205	0.1–5	16.552	259	205	0.1–5
Haloperidol	16.777	224	237	0.5–5	16.779	224	237	0.1–5
Alprazolam	16.872	204	308	0.1–5	16.876	204	308	0.05–5
Paroxetine	—				16.999[a]	234	371	0.01–5
Hydroxyzine	16.138	201	165	0.1–5	17.066[a]	201	165	0.1–5
Meclizine	17.314	105	189	0.05–5	17.323	105	189	0.01–5
Sultopride	17.54	98	134	0.1–5	17.554	98	134	0.1–5
Etizolam	17.628	342	266	0.1–5	17.64	342	266	0.01–5
Floropipamide	17.65	165	331	0.5–5	17.66	165	331	0.1–5
Triazolam	17.728	313	238	0.1–5	17.73	313	238	0.1–5
Bromperidol	17.794	268	281	0.5–5	—			
Amoxapine	14.873	245	257	0.05–5	17.867[a]	257	355	0.01–5
Brotizolam	18.01	245	394	0.1–5	18.014	245	394	0.1–5
Thioridazine	18.371	98	370	0.05–5	18.379	98	370	0.01–5
Fluphenazine	—				18.545[a]	280	248	0.5–5
4-Hydroxyalprazolam	—				18.565[a]	295	281	0.5–5
Verapamil	18.676	303	58	0.1–5	18.683	303	58	0.1–5
4-Hydroxytriazolam	—				19.547[a]	329	315	0.5–5
1-Methylhydroxyetizolam	—				19.906[a]	357	400	0.1–5
α-Hydroxytriazolam	—				19.946[a]	357	400	0.1–5
α-Hydroxybrotizolam	—				20.542[a]	409	452	0.1–5
Quetiapine	—				21.234[a]	210	186	0.1–5
Nemonapride	21.69	173	198	0.1–5	21.681	173	198	0.5–5
Propericiazine	20.218	114	365	0.5–5	21.878[a]	156	184	0.05–5
Oxypertine	22.816	175	204	0.5–5	21.879[a]	175	204	0.1–5
Perphenazine	—				21.880[a]	246	445	0.5–5

MDA, 3,4-Methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethampheamine; MBDB, *N*-methylbenzodioxazolylbutanamine; 2C-B, 4-bromo-2,5-dimethoxyphenetylamine; 2C-I, 4-iodo-2,5-dimethoxyphenethylamine; 2C-T-2, 2,5-dimethoxy-4-ethylthio-β-phenethylamine; 2C-T-7, 2,5-dimethoxy-4-(*n*)-propylthiophenethylamine

[a] Analyzed as the acetate

[b] Artifact

[c] Artifact acetate

provided by Nishikawa Keisoku. An HP-5ms fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness, Agilent) coated with 5% phenylmethylsilicone stationary phase was used. The splitless injection mode was selected with a valve-off time of 2 min. The GC-MS conditions were as follows. The oven temperature was initially 60°C, held at the temperature for 2 min, programmed to 300°C at 20°C/min and held at 300°C for 10 min. The total run time was 24 min. The injection port and transfer line temperatures were 250°C and 280°C, respectively. The carrier gas was helium and the constant pressure mode was used. Decafluorotriphenylphosphine (DFTPP) tuning was carried out to obtain a uniform mass spectrum. The retention times were fixed using the retention-time locking (RTL) technique with diazepam-d_5 as the locking compound. We set the retention time of diazepam-d_5 at 13.923 min. The full-scan mode (scanning range 50–550 amu) was used.

Registration of each data for the "free-drug database" and "drug database with acetylating reagents"

One quantifier (target) ion and one qualifier ion were selected for each drug and a calibration curve was obtained by plotting the peak area ratio of the drug to the IS versus the amount of drug using MSD ChemStation D.02.00.275 (Agilent). The retention time and mass spectrum of each drug were obtained at the level of 1 μg/ml (20 ng on column). The data obtained for each drug, such as retention time, qualifier ion/target ion (QT) percentage, mass spectrum, and calibration curve (*a* and *b* for a linear curve, and *a*, *b*, and *c* for a quadratic curve), were registered using the NAGINATA software as "free-drug database" and "drug database with acetylating reagents."

Analysis of spiked samples using the "drug database with acetylating reagents"

Forty drugs most commonly encountered in toxicological cases in Japan were selected on the basis of statistical data of fatal poisoning [10], and the applicability of the constructed database was examined by analyzing whole blood samples spiked with these drugs. Forty drugs shown in Table 2 were divided into four groups (each containing ten drugs) and a standard mixture solution of each group was made by dissolving these drugs in methanol. We prepared whole blood samples ($n = 2$) spiked with ten drugs for each group at low (0.1 μg/ml) and high (1 μg/ml) concentrations. The samples were extracted with the Focus columns and incubated with the acetylating reagents according to our method [11] with slight modification. To 1.0 ml of a whole blood sample, 300 μl of 1 M acetate buffer (pH 5.0) and 2 ml of distilled water were added. The mixture was vortex-mixed for 30 s, sonicated for 5 min and centrifuged at 850 *g* for 15 min. The supernatant was applied to a Focus column previously conditioned sequentially with 1 ml of methanol and 1 ml of distilled water. The column was rinsed with 1 ml of distilled water and 1 ml of 10% acetonitrile (ACN). The analytes were eluted with 0.75 ml of 0.1% trifluoroacetic acid in ACN solution and then 0.75 ml of 0.2% ammonia in ACN solution. The eluate was evaporated to dryness under a stream of nitrogen at 60°C. The residue was dissolved in 50 μl of pyridine, and 50 μl of acetic anhydride was added to the solution to carry out the acetylation. The mixture was incubated at 60°C for 30 min, and then the solvent was evaporated to dryness at room temperature. The residue was dissolved in 100 μl of ethyl acetate containing 1 μg of IS, and a 2-μl aliquot of the solution was injected into the GC-MS apparatus. Then, the NAGINATA screening was carried out using our newly developed "drug database with acetylating reagents." The scheme of the screening method using NAGINATA software was previously mentioned [9].

The recovery of each drug at the level of 1 μg/ml was also obtained by comparing the peak area of each drug in a whole blood extract with that in the standard solution (Table 2).

Practical application

The established screening method using the "drug database with acetylating reagents" was applied for whole blood and urine samples in forensic autopsy cases carried out at our department. The whole blood samples were extracted as described above. The urine sample was extracted as follows; 1 ml of urine sample was mixed with 100 μl of 1 M acetate buffer (pH 5) and was hydrolyzed by 50 μl of β-glucuronidase (Type HP-2, Sigma-Aldrich) at 37°C for 2 h. To this solution, 1 ml of distilled water was added, and the mixture was vortex-mixed for 30 s, sonicated for 5 min, and centrifuged at 850 *g* for 15 min. The supernatant was applied to the Focus column, incubated with the acetylating reagents, and subjected to GC-MS as described for the spiked whole blood samples. The NAGINATA screening was also carried out by our "drug database with acetylating reagents," comparing with the conventional screening using NIST and PMW_TOX mass spectra libraries.

Table 2 Results of NAGINATA screening using "drug database with acetylating reagents" for whole blood samples spiked with 40 drugs

Drug name	Therapeutic category	Retention time (min)	0.1 μg/ml		1 μg/ml		
			Drug confirmation	Tentative quantification value (μg/ml)	Drug confirmation	Tentative quantification value (μg/ml)	Recovery (%)
Bromisovalum	Hypnotic	6.111	–		+	0.045	4.8
Zolpidem	Hypnotic	15.786	+++++	0.005	+++++	0.395	27.6
Amobarbital	Hypnotic	10.008	+++++	0.065	+++++	0.81	76.1
Pentobarbital	Hypnotic	10.168	+++++	0.105	+++++	0.935	59.9
Phenobarbital	Hypnotic	11.485	+++++	0.5	+++++	0.83	78.7
Alprazolam	Hypnotic	16.87	+++++	0.075	+++++	0.195	28.7
Brotizolam	Hypnotic	18.011	+	0.09	+++++	0.275	20.8
Diazepam	Hypnotic	13.927	+	0.06	+++++	0.54	62.4
Estazolam	Hypnotic	16.554	+	0.08	+++++	0.345	52.7
Etizolam	Hypnotic	17.623	++++	0.05	+++++	0.165	20.4
Flunitrazepam	Hypnotic	14.754	+	0.13	+++++	0.27	25.5
Flurazepam	Hypnotic	15.548	+++++	0.07	+++++	0.505	60.5
Midazolam	Hypnotic	14.638	++++	0.07	+++++	0.305	37.2
N-Desmethyldiazepam	Hypnotic	14.227	+		+++++	0.64	23.7
Triazolam	Hypnotic	17.721	++++	0.14	+++++	0.385	28.8
Amitriptyline	Antidepressant	12.739	++++	0.065	+++++	0.5	56.8
Amoxapine[a]	Antidepressant	17.873	+++++	0.065	+++++	0.355	36.0
Clomipramine	Antidepressant	13.76	+++++	–0.02	+++++	0.68	69.0
Fluvoxamine[a]	Antidepressant	12.819	+++++	0.055	+++++	0.215	21.0
Imipramine	Antidepressant	12.879	+++++	0.02	+++++	0.555	63.4
Paroxetine[a]	Antidepressant	16.983	+++++	0.045	+++++	0.285	34.8
Chlorpromazine	Antipsychotic	14.183	+++++	0.035	+++++	0.285	28.6
Haloperidol	Antipsychotic	16.8	–		+	0.125	7.5
Levomepromazine	Antipsychotic	14.293	+++++	0.04	+++++	0.35	37.6
Olanzapine[a]	Antipsychotic	15.602	+	0.05	+++++	0.06	12.2
Promethazine	Antipsychotic	13.175	+++++	0.07	+++++	0.4	38.1
Carbamazepine	Antiepileptic	13.386	+++++	0.08	+++++	0.73	69.5
Biperiden	Antiparkinson	13.179	+++++	0.06	+++++	0.665	60.7
Chlorpheniramine	Antihistamine	11.744	+++++	0.07	+++++	0.665	70.7
Diphenhydramine	Antihistamine	10.958	++++	0.12	+++++	0.94	93.1
Lidocaine	Local anesthetic	11.034	++++	0.085	+++++	0.775	84.2
Acetaminophen[a]	Antipyretic analgesic	10.344	+	0.04	+++++	0.045	8.8
Caffeine	Antipyretic analgesic	10.75	+	0.035	+++++	0.075	6.7
Ibuprofen	Antipyretic analgesic	9.303	+++++	0.53	+++++	0.565	146.8
Dihydrocodeine[a]	Antitussive	14.1	+++++	0.075	+++++	0.74	79.1
Ephedrine[a]	Antitussive	10.565	++++	0.055	+++++	0.405	55.1
Amphetamine[a]	Abused drug	8.66	+++++	0.1	+++++	1.01	112.2
Methamphetamine[a]	Abused drug	9.223	+++++	0.19	+++++	1.38	103.4
MDMA[a]	Abused drug	11.35	+++++	0.085	+++++	0.735	76.6
β-Phenethylamine[a]	Putrefied amine	8.625	+++++	0.155	+++++	1.065	88.4
			Average	0.096	Average	0.51	51.7

[a] Analyzed as the acetylated form

Results and discussion

Construction of drug databases

When we injected 210 drugs into the GC-MS in their free forms at the level of 1 μg/ml, only 133 drugs were detectable. In our previous study for analyzing drugs of abuse, acetylation was found to be most suitable for derivatization in their analysis by GC-MS [12]. Thus, we tried to analyze the 210 drugs after incubation with acetylating reagents, and a significant improvement was observed in the number of drugs detected; 166 compounds became detectable with acetylation or only with addition of the acetylating reagents. In the 166 drugs, 72 drugs were acetylated; 36 drugs such as morphine and benzodiazepine metabolites became detectable only by acetylation, and the other 36 drugs such as maprotiline and pentazocine showed significant increases in sensitivity (>ten times) as compared with the free drugs. Furthermore, the addition of the reagents improved the stability of 26

drugs such as antipsychotics and antidepressant drugs, and gave twofold to fivefold increase in sensitivity, although the drugs were not acetylated. Saka et al. [13] found that the addition of acetic acid to the solvent improved the sensitivity of theophylline by preventing the drug from adsorbing onto the glass wool packed in the inlet liner. A similar mechanism may take place by using pyridine and acetic anhydride in our procedure.

For the analysis of pesticide and environmental compounds using the preset "pesticide database" in the NAGINATA software, five deuterated aromatic compounds were added to the extract as ISs at the final step before GC-MS analysis. These ISs are useful to some extent to estimate the concentration of the above compounds in the materials, but are not suitable for final quantitation because they are not added at the initial step, and have completely different structures from the target compounds. To overcome such a problem, a deuterated benzodiazepine drug, diazepam-d_5, was selected as IS in our procedure. We could successfully determine the concentration of a drug detected by our NAGINATA screening using the same diazepam-d_5 as IS by adding it again at the initial step of extraction (data not shown).

After careful examination of sensitivity and correlation coefficient of calibration curve for each compound, 127 drugs and 156 drugs were selected to construct a "free-drug database" and a "drug database with acetylating reagents," respectively. Table 1 shows the list of drugs in the constructed databases with each retention time, target ion, qualifier ion, and calibration range. Because the "drug database with acetylating reagents" contains more drugs and can cover lower ranges of concentration in many drugs than the "free-drug database," the former database is considered to be superior for drug screening in forensic cases. The "free-drug database" may be useful for quick screening in emergency cases, because no derivatization step is required.

Analysis of spiked samples using the "drug database with acetylating reagents"

Table 2 shows the results of our NAGINATA screening for whole blood samples spiked with 40 drugs most commonly encountered in toxicological cases in Japan. Among the 40 drugs examined, 38 were successfully identified showing ++++ or +++++ marks at 1.0 μg/ml and 30 drugs were similarly identified at 0.1 μg/ml. The absolute recoveries of drugs using the Focus column at the concentration of 1 μg/ml was more than 70% in 11 drugs, 50%–70% in 10 drugs, 20%–50% in 14 drugs, and less than 20% in 5 drugs. Because the drugs such as caffeine and acetaminophen showed poor recoveries by the Focus column, another extraction procedure should be explored for identifying these drugs at lower concentrations.

Practical application

Figure 1 shows a typical Quant Screener Report (QSR) by our NAGINATA screening method using the "database with acetylating reagents" for a whole blood sample in an autopsy case. This report includes: (1) the actual and expected retention times in the database and their difference, (2) the target ion abundance, (3) the actual and expected QT percentages, (4) the agreement between the actual and expected mass spectra, (5) the tentative quantitation values, and (6) the drug confirmation degrees (from no mark to +++++ mark). Therefore, the probability of drug existence in a sample was quickly estimated by looking at this QSR report. Another merit of using this software is that the presence of a drug can be manually confirmed using the NAGINATA browser as explained in the previous report [9]. By clicking the name of drug on the screen, all data corresponding to the drug such as mass chromatograms and a total ion chromatogram, actual and expected mass spectra of the peak, and a calibration curve can be presented on one screen. Therefore, an analyst can conveniently make a judgment of the analysis with the NAGINATA browser.

Because each drug always gives the same retention time by the RTL technique, further screening using commercially available mass spectral libraries is much easier, and useful information on other drugs and metabolites can be obtained especially for urine samples by the PMW_TOX library. If the recovery of each drug is known in advance, semiquantitative data obtained by our NAGINATA screening become very useful to set the calibration range for the next quantitation step. The constructed databases have been used in three different laboratories so far, and given the same reliable data in each laboratory, which will be presented elsewhere. Therefore, our software-assisted toxicological screening has proven to be very useful in toxicological analysis.

Conclusions

We have constructed unique calibration-locking databases for rapid and semiquantitative drug screening by GC-MS using NAGINATA, a novel GC-MS software package. The time for data analysis was less than 1 min, and semiquantitative data could be obtained without preparing standard compounds. Because the constructed databases can be used in any laboratory, and the drug items can be easily added, the present screening proce-

Fig. 1 An example of a Quant Screener Report by the NAGINATA screening

Quant Screener Report

Acquisition Date	18 Jun 2008 14:03
Data File	C:¥MSDChem¥1¥DATA¥NAGINATA¥08June18¥0830SBL.D
Method File	C:¥MSDChem¥1¥METHODS¥mix9free.M
Sample Name	0830SBL
Misc Info.	
Vial Number	1
Operator	kudo
Analysis Date	Wed Nov 19 09:52:32 2008
Screening File	C:¥Chemplus¥QSR¥File¥Data¥STD-AC08.qsd
Analysis File	C:¥MSDChem¥1¥DATA¥NAGINATA¥08June18¥0830SBL.D¥QSR¥STD-AC08.daa
NAGINATA Tuning Date	
System Performance Check Date	
System Performance Check Data	

Analysis Result 157 Compounds
Result ++++ Over 8 Compounds
Result ++ Over 0 Compounds

Compound	RT[min] Actual	RT[min] DB	RT[min] Dif[sec]	Area	QT[%] Actual	QT[%] DB	MS Hit	Tentative Quant[μg/ml]	Result
(IS)Diazepam-D5 [STD-AC]	13.92	13.92	−0.33	879489	74.10	73.87	95	1.00	+++++
Carbamazepine [STD-AC]	13.41	13.39	1.35	10020256	26.65	25.72	96	5.34	+++++
Levomepromazine [STD-AC]	14.28	14.29	−0.87	631096	16.56	15.40	99	0.18	+++++
Lidocaine [STD-AC]	11.02	11.04	−1.33	1364611	9.43	7.13	76	0.30	+++++
Mephobarbital [STD-AC]	11.15	11.17	−1.13	74451	36.52	40.40	95	0.11	+++++
Phenobarbital [STD-AC]	11.51	11.50	0.86	109163	14.15	11.59	93	0.68	+++++
Quetiapine AC [STD-AC]	21.21	21.23	−1.43	114125	50.17	56.67	99	0.36	+++++
Sultopride [STD-AC]	17.59	17.55	1.96	454335	4.00	2.61	64	0.23	++++
1-Methylhydroxyetizolam [STD-A	20.03	19.91	7.61	883	0.00	42.81	0		+
2C-T-2 AC [STD-AC]	13.26	13.17	5.17	1313	0.00	18.92	0		+
2C-T-7 AC [STD-AC]	13.50	13.53	−1.67	1043	0.00	18.33	0		+
4-aminoantipyrine AC [STD-AC]	13.04	13.07	−1.74	8598	0.00	34.12	0		+
4MPP AC [STD-AC]	12.62	12.64	−1.08	1952	0.00	76.76	0		+
4-MTA AC [STD-AC]	11.54	11.34	11.99	479771	16.61	22.00	0		+
4OH-AP 2AC [STD-AC]	11.02	11.03	−0.67	11831	0.00	68.55	0		+
4OH-MA 2AC [STD-AC]	11.66	11.49	9.96	3369	49.76	41.70	0		+
5MeO-AMT AC [STD-AC]	13.61	13.43	10.83	4312	39.47	65.55	0		+
5MeO-DMT [STD-AC]	11.66	11.71	−3.06	3948	0.00	5.16	0		+
5MeO-MIPT [STD-AC]	12.48	12.49	−0.30	1569	0.00	4.77	0		+
Acetylpheneturide-arti [STD-AC]	8.51	8.52	−1.02	8798	14.74	19.74	0		+
Alfa-hydroxytriazolam AC [STD-A	20.03	19.95	5.21	2333	0.00	8.15	0		+
Allylisopropylacetylurea [STD-AC	6.36	6.37	−0.53	1944	0.00	20.93	0		+
Alprazolam [STD-AC]	17.02	16.88	8.69	2400	0.00	60.10	0		+
Amitriptyline [STD-AC]	12.59	12.74	−9.31	23209	0.00	3.47	45		+
Amoxapine AC [STD-AC]	17.86	17.87	−0.36	1690	38.20	23.11	0		+
Amphetamine AC [STD-AC]	8.60	8.70	−5.69	4460	0.00	67.21	9		+
AMT AC [STD-AC]	12.35	12.44	−5.60	1764	78.48	77.91	0		+
Antipyrine [STD-AC]	10.84	11.00	−9.84	2355	108.67	65.75	0		+
Atropine AC [STD-AC]	13.23	13.23	−0.36	2712	0.00	17.56	0		+
Barbital [STD-AC]	8.45	8.59	−8.42	4740	0.00	101.13	0		+
Beta-phenethylamine AC [STD-A	8.81	8.64	10.24	3151	0.00	18.11	0		+
Biperiden [STD-AC]	13.05	13.18	−8.12	17510	13.21	13.17	0		+
Bisacodyl [STD-AC]	15.86	15.87	−0.96	8999	69.71	67.64	0		+
Bromazepam [STD-AC]	14.89	14.83	3.83	14262	0.00	0.00	0		+
Bromisovalum [STD-AC]	6.03	6.13	−5.68	2971	45.31	101.99	0		+
Brotizolam [STD-AC]	17.98	18.01	−1.98	817	0.00	94.06	0		+
Caffeine [STD-AC]	10.74	10.76	−1.04	50897	50.88	52.05	0		+
Chlordiazepoxide-arti [STD-AC]	14.28	14.25	1.41	32168	5.18	23.35	0		+
Chlormezanone [STD-AC]	13.17	13.06	6.71	12392	47.31	87.43	0		+
Chlorpheniramine [STD-AC]	11.56	11.75	−11.64	9543	38.81	54.26	0		+
Chlorpromazine [STD-AC]	14.17	14.19	−0.96	10133	0.00	14.05	0		+
Clomipramine [STD-AC2]	13.71	13.80	−5.46	49354	2.09	42.69	0		+

dure using the databases seems to be very useful in clinical and forensic toxicological analyses.

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