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Systematic Toxicological Analysis Using HPLC/DAD

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ABSTRACT: A high-performance liquid chromatographic method with diode-array detection (HPLC/DAD) for systematic toxicological analysis of human blood or plasma samples is presented. After single-step liquid/liquid extraction at pH 9.5 using chloroform/2-propanol/*n*-heptane (60:14:26, v/v/v), the drugs elute isocratically from a NovaPak C18 (Waters) 4-μm column (300 mm × 3.9 mm, i.d.) at 30°C, with methanol/tetrahydrofuran/pH 2.6 phosphate buffer (65:5:30, v/v/v) as the mobile phase (flow rate 0.8 mL/min). Full UV spectra from 200 to 400 nm (resolution 1.3 nm) are recorded on-line during the 20 min chromatographic run. Solute identification may be automatically performed by comparison of analytical data (retention times and UV spectra) with references of 311 pharmaceuticals, toxicants and drugs of abuse stored in a computerized library. The method is simple, rapid, relatively inexpensive and highly specific. The previously reported applications of HPLC/DAD technology to drug screening are reviewed, and the interests and limitations of the method are discussed in the light of this literature.

KEYWORDS: toxicology, HPLC, diode array, systematic toxicological analysis, screening

Acute poisonings account for 10 to 30% of the admissions to nonspecialized intensive care units, and 7 to 15% of autopsies performed in forensic institutions. Consequently, the development of reliable techniques for "general-unknown" screening (or systematic toxicological analysis, STA) of pharmaceuticals, toxicants, and drugs of abuse in biological fluids constitutes a necessary work load for every laboratory dealing with forensic toxicology. Such screening methods should be simple, rapid, reproducible, and able to cover a broad spectrum of toxicologically relevant substances, in one analytical run, with sufficient specificity. Numerous procedures have been proposed for such purposes, including colorimetric determinations, UV spectrophotometry, immunoassays, thin-layer chromatography, gas chromatography (GC) coupled to classical or mass spectrometric (MS) detectors, as well as normal- or reverse-phase high-performance liquid chromatography (HPLC). Among these methods, the coupling of HPLC to diode-array detection (HPLC/DAD) has been gaining more importance for some years, and is considered as a promising tool in this area of analytical toxicology. As an illustration, we present a highly specific HPLC/DAD method allowing simple and rapid identification of more than 300 compounds in human blood or plasma.

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Materials and Methods

Materials

Methanol, chloroform, tetrahydrofuran, 2-propanol, and *n*-heptane were HPLC grade (Merck, F.R.G.); all other chemicals and reagents were of analytical grade and purchased from Merck or Prolabo (France).

Most drug standards used for the library were generously donated by their respective manufacturers. Other standards (especially non-pharmaceuticals) were obtained from Merck (F.R.G.) or Aldrich (France). For each drug, stock solutions at 100.0 μg/mL were prepared in methanol, stored at 4°C in the dark, and controlled monthly for stability; work solutions at 10.0 and 1.0 μg/mL were prepared daily by appropriate methanolic dilutions.

The pH 2.6 buffer was prepared with a 10^{-2} M (0.68 mg/mL) KH_2PO_4 solution, adjusted to the desired pH by appropriate addition of concentrated orthophosphoric acid. The pH 9.5 buffer was prepared using a saturated (25 to 28% w/w at ambient temperature) NH_4Cl solution, 25% diluted with deionized water, and adjusted to the desired pH by appropriate addition of 25% ammonia solution.

Chromatography

All analyses were performed on a Waters (Millipore Corp., U.S.A.) HPLC apparatus, consisting of a quaternary low-pressure pump (Waters mod. 600 E), a 200-μL loop volume autoinjector with 48-vial carousel (Waters mod. 715 UltraWisp), and a UV-VIS diode-array spectrophotometer (Waters mod. 991) with 512 diodes, 0.15-mm slit, 8.0-μL flow cell, 10.0-mm optical path length, a wavelength range from 190 to 800 nm, and maximal spectral resolution of 1.3 nm.

The system was monitored by a computer (PowerMate SX Plus, NEC, U.S.A.) with software (Waters PDA) allowing the creation of a personal database, and automatic comparison of current analytical data (retention times and UV spectra) with references previously stored in this library. Drug identification was carried out, first using a *time window* parameter that restricts the library search to a definite slice around the retention time of an unknown peak, then by point-to-point comparison of the unknown spectrum to spectra of all reference compounds comprised within this window, calculation in each case of a fit value (degree of similarity) ranging from 0 to 1000, and listing of all compounds exhibiting fit values higher than a predefined *fit threshold.* Time window and fit threshold were set at ±0.5 min (±1.0 min for compounds eluted after 9 min) and 900/1000 (a value low enough to take account of minor

changes in UV spectra at different solute concentrations), respectively.

The column was a NovaPak C18 (Waters) 4 μm, pore size 6 nm (300 × 3.9 mm, i.d.), thermostated at 30°C during all experiments.

The elution was achieved isocratically (flow rate 0.8 mL/min, average operating pressure 19.65 MPa) with a mobile phase of methanol/tetrahydrofuran/pH 2.6 phosphate buffer (65:5:30, v/v/v). At the beginning of each chromatographic session, this mobile phase was degassed and filtered through 0.45-μm filters (Durapore GVWP 047, Millipore Corp., U.S.A.) with a Pyrex filter holder (Millipore, U.S.A.). After 30-min equilibration of the chromatographic system, the outlet of the DAD was then plugged into the mobile phase reservoir bottle for solvent recycling; at the end of the session, the system was opened and the column was washed with deionized water (0.8 mL/min for 1 h) then methanol (0.8 mL/min for 1 h).

Procedure

To 2.0 mL blood or plasma in a 15-mL Pyrex centrifuge tube were added 2.0 mL of the pH 9.5 ammonium chloride buffer and 5.0 mL of the extracting solvent (chloroform/2-propanol/*n*-heptane, 60:14:26, v/v/v). The mixture was gently shaken on a horizontal agitator for 10 min, then centrifuged at 2800 *g* for 10 min. The lower organic phase was removed and evaporated to dryness at 45°C in a rotary evaporator (Speed Vac Concentrator mod. A290, Savant Instruments, U.S.A.). The residue was dissolved in 100 μL of the mobile phase; after centrifugation (2800 *g* for 5 min), 50 μL of the supernatant were then injected onto the column. The duration of the chromatographic run was set at 20 min. The eluent was monitored at 210 nm, while full UV spectra (200 to 400 nm) were recorded on-line during the whole chromatographic run. The spectral resolution and sampling interval (delay between acquisition of two successive spectra) were set at 1.3 nm and 1.0 s, respectively.

Results

To date, the present method allows easy and rapid identification, in plasma or whole blood, of 311 substances belonging to 22 different pharmacological classes: benzodiazepines, metabolites and antagonists (34), barbiturates (4), antidepressants (29), neuroleptics (33), opiates, metabolites, antagonists and morphinomimetic synthetic derivatives (15), psychostimulants (5), cyclopyrrolones (2), imidazopyridines (5), beta-blockers (18), calcium channel blockers (9), vasodilators, anti-arrhythmic and antihypertensive agents (26), antihistamines H1 (20), non-steroidal anti-inflammatory agents (24), other analgesics (8), antidiabetic agents (6), antiparasitics (9), antineoplastic agents (13), anti-ulcer agents (5), beta-stimulants (3), local anaesthetic agents (6), anticoagulants and inhibitors of platelet aggregation (7), and miscellaneous drugs (30). The retention data and main UV absorption peaks of the library's reference compounds are listed in Table 1.

The day-to-day variability of retention times (RTs) has been estimated using a series of 20 compounds frequently involved in acute poisonings (viloxazine, clomipramine, sulpiride, trifluoperazine, bromazepam, prazepam, phenobarbital, morphine, dextropropoxyphene, atenolol, betaxolol, diphenhydramine, benzoylecgonine, verapamil, ketoprofene, zopiclone, zolpidem, paracetamol, chloroquine, and lidocaine): 10.0 μg/mL and 1.0 μg/mL methanolic solutions of each drug have been assayed weekly in triplicate over a 3-months period; between two successive assays, routine analyses (about 50/week) of clinical or forensic samples were

TABLE 1—*Retention and spectral data for 311 pharmaceuticals, toxicants and drugs of abuse.*

Compound	RT (min)	k′	λ_{max}(nm)
Tenoxicam[a]	2.99	0.01	264; **376**
Iproniazide	3.00	0.01	**264**
Methocarbamol	3.01	0.01	**223;** 274
Molsidomine	3.02	0.01	229; **314**
7-Aminoclonazepam[a]	3.02	0.01	**240;** 352
Ethyle biscoumacetate	3.04	0.02	**275;** 304
Methotrexate	3.06	0.03	243; **301**
Caffeine[a]	3.06	0.03	**273**
Nialamide	3.07	0.03	**264**
Colchicine	3.07	0.03	**244;** 352
Cytarabine	3.10	0.04	**278**
Benzoylecgonine[a]	3.10	0.04	**231;** 274
Paracetamol[a]	3.10	0.04	**247**
Diazoxide	3.13	0.05	**265**
7-Aminoflunitrazepam[a]	3.16	0.06	**242;** 348
Dacarbazine	3.16	0.06	235; **325**
7-Aminonitrazepam[a]	3.18	0.08	**242;** 349
Thiopental metabolite[a]	3.25	0.09	238; **288**
Sulfinpyrazole	3.25	0.09	**261**
Flumazenil[a]	3.26	0.09	**244**
Sulpiride[a]	3.26	0.09	**292**
Morphine[a]	3.32	0.11	**287**
Atenolol[a]	3.33	0.12	**225;** 274; 283
Toloxatone[a]	3.35	0.12	**238;** 272; 281
Terbutaline	3.36	0.13	**225;** 278
Salbutamol[a]	3.37	0.13	**226;** 277
Phenobarbital[a]	3.38	0.13	n.p.
Ranitidine	3.38	0.13	229; **322**
Tiapride[a]	3.38	0.13	n.p.
N-Desmethylnizatidine	3.38	0.13	249; **322**
Etodolac metabolite 3	3.40	0.14	223; **277**
Phenol	3.40	0.14	**272**
Chlormenazone	3.40	0.14	**223;** 260; 265
Acetylsalicylic acid[a]	3.40	0.14	**233;** 300
Metformine	3.41	0.14	**231**
3,4-Diaminobenzophenone[a]	3.42	0.15	**251;** 355
Ritodrine	3.43	0.15	**225;** 275
Hydroxyglafenic acid[a]	3.43	0.15	**226;** 249; 379
Codeine[a]	3.44	0.15	**286**
Sultopride[a]	3.47	0.16	**235;** 287
6-Monoacetylmorphine[a]	3.48	0.17	**285**
Amisulpride[a]	3.48	0.17	**225;** 281
Nizatidine sulfoxide	3.48	0.17	251; **324**
Naltrexone	3.49	0.17	**225;** 283
Hydroxyfloctafenic acid	3.49	0.17	**379**
Lisinopril	3.50	0.17	**259**
Benzocaine	3.51	0.18	221; **291**
Nizatidine	3.52	0.18	255; **322**
Nalorphine	3.52	0.18	**287**
Mephenesine	3.53	0.18	**272;** 278
Naloxone	3.56	0.19	**283**
Sotalol[a]	3.58	0.20	**229;** 270
Carteolol[a]	3.60	0.21	**251;** 279
Glafenic acid[a]	3.61	0.21	**226;** 369
Hydroxydebrisoquine	3.62	0.21	**261**
Alizapride	3.65	0.22	**223;** 296
Codethyline[a]	3.65	0.22	**286**
Linsidomine	3.65	0.22	255; **294**
Procainamide	3.65	0.22	**287**
4-Nitrophenol	3.66	0.23	226; **316**
Carbamazepine	3.67	0.23	238; **283**
Bromazepam[a]	3.67	0.23	**234;** 318
Nalbuphine	3.67	0.23	**259**
Nadolol	3.69	0.24	**270;** 277
Procarbazine	3.69	0.24	**231**
Etodolac metabolite 2	3.70	0.24	**223;** 272; 296
Dihydralazine[a]	3.70	0.24	**260;** 305
Omeprazole	3.72	0.25	277; **303**
Strychnine	3.77	0.27	**255**

(*continued*)

TABLE 1—*Continued.*

Compound	RT (min)	k′	λ_{max}(nm)
Acebutolol[a]	3.80	0.28	**235;** 327
Bromazepam metabolite[a]	3.83	0.29	**238**
Gluthetimide	3.83	0.29	**259**
Chlorpropamide	3.85	0.29	**231;** 265
Desmethylclobazam	3.85	0.29	**229;** 292
Clomethiazole	3.85	0.29	**249**
Glipizide	3.86	0.30	**226;** 275
Mitoguazone	3.87	0.30	**285**
Fluindione	3.87	0.30	**223;** 275; 333; 343
Triazolam	3.88	0.30	**222**
Prazosine	3.89	0.31	**247;** 330
Tryptamine[a]	3.89	0.31	**279;** 288
Flunitrazepam[a]	3.92	0.32	**221;** 253; 311
Clonazepam[a]	3.92	0.32	**246;** 311
Metoclopramide[a]	3.92	0.32	**275;** 311
Urapidil	3.93	0.32	238; **268**
Melphalan	3.93	0.32	**261;** 302
Estazolam	3.94	0.32	**223**
Tolbutamide	3.95	0.33	**229;** 264
Ephedrine	3.95	0.33	**259**
Clonidine	3.96	0.33	**272**
Pindolol	3.98	0.34	**265;** 287
Clobazam[a]	4.00	0.34	**230;** 291
Minoxidil	4.01	0.35	**231;** 281
Disopyramide[a]	4.01	0.35	**261**
Mefloquine metabolite	4.02	0.35	**223;** 290
Nitrazepam[a]	4.03	0.35	**260;** 309
Dextromethorphane	4.03	0.35	**220;** 281
Tofisopam	4.04	0.36	**238;** 270; 311
Zopiclone[a]	4.05	0.36	**305**
Debrisoquine	4.07	0.37	**262**
Sulindac	4.11	0.38	**227;** 286; 329
Alprazolam[a]	4.17	0.40	**222**
Cycloguanil	4.19	0.41	**243**
Lorazepam[a]	4.19	0.41	**230;** 318
Methaqualone	4.19	0.41	**226;** 265; 305; 317
Ketamine	4.19	0.41	**269;** 277
Piroxicam[a]	4.20	0.41	243; **338**
Metoprolol	4.20	0.41	**223;** 274; 283
Nifedipine[a]	4.21	0.41	**236;** 339
Quinine[a]	4.28	0.44	**233;** 281; 334
Mephentermine	4.30	0.44	**259**
Prilocaine	4.32	0.45	**227**
Pentazocine	4.33	0.45	**220;** 281
4-Chlorophenol	4.33	0.45	**225;** 282
Oxazepam[a]	4.34	0.46	**229;** 314
Tiaprofenic acid[a]	4.34	0.46	265; **307**
Quinidine[a]	4.34	0.46	**233;** 281; 334
Oxomemazine	4.36	0.46	**226;** 268; 294; 331
Celiprolol	4.36	0.46	**233;** 330
Ajmaline	4.37	0.47	**246;** 291
Alpidem metabolite 1[a]	4.37	0.47	**234;** 318
Ketotifene	4.40	0.48	**299**
Yohimbine	4.40	0.48	**272;** 288
Tolonidine	4.40	0.48	n.p.
Lidocaine[a]	4.45	0.49	**264**
Secobarbital[a]	4.48	0.50	n.p.
Viloxazine[a]	4.49	0.51	**222;** 274
Metopimazine	4.50	0.51	221; **264;** 312
Mepivacaine	4.54	0.52	**264**
Pethidine	4.55	0.53	**259**
Doxylamine[a]	4.57	0.53	**261**
Labetalol	4.58	0.54	**229;** 304
Temazepam[a]	4.58	0.54	**230;** 313
Bretazenil	4.60	0.54	n.p.
Amodiaquine	4.62	0.55	**225;** 238; 344
Benperidol	4.62	0.55	231; **246;** 279
Droperidol[a]	4.65	0.56	230; **246;** 279
Hydroxychloroquine	4.65	0.56	**222;** 236; 257; 331; 344

TABLE 1—*Continued.*

Compound	RT (min)	k′	λ_{max}(nm)
Zolpidem[a]	4.66	0.56	**239;** 298
Ketoprofene[a]	4.69	0.57	**257**
Alminoprofene	4.69	0.57	**253;** 298
Cicletanine	4.70	0.58	**220;** 285; 316
Moclobemide	4.71	0.58	**239**
Chloroquine[a]	4.72	0.58	**222;** 236; 257; 331; 344
Cocaine	4.75	0.59	**231;** 275
Timolol[a]	4.76	0.60	**299**
Etodolac metabolite 1	4.79	0.61	**225;** 281
Nomifensine	4.87	0.63	**240;** 290
Floctafenic acid[a]	4.89	0.64	249; **368**
Ticlopidine	4.91	0.65	**234;** 269; 275
Acenocoumarol	4.91	0.65	**283;** 305
Vindesine	4.92	0.65	**270;** 310
Imolamine	4.95	0.66	**229**
Mexiletine	4.95	0.66	**262**
Dipyridamole[a]	4.97	0.67	231; **285**
Trazodone[a]	4.98	0.67	**249;** 277; 318
Pipamperone	4.98	0.67	**246**
Pyrimethamine[a]	5.00	0.68	**273**
Benazepril	5.01	0.68	**239**
Vincristine	5.06	0.70	**221;** 257; 298
Azatadine	5.06	0.70	**235;** 270
Metapramine[a]	5.08	0.70	246; **269**
Chlordiazepoxide	5.10	0.71	247; **262**
Oxprenolol[a]	5.18	0.74	222; **273**
Warfarine	5.18	0.74	273; **283;** 305
Clorazepate	5.19	0.74	**229;** 316
Desmethyldiazepam[a]	5.20	0.74	**229;** 314
Flecainide[a]	5.22	0.75	**299**
Nadoxolol	5.22	0.75	**291;** 320
Phencyclidine	5.22	0.75	**262;** 270
Fluanisone	5.24	0.76	**244**
Thiopental[a]	5.26	0.77	238; **287**
Diltiazem metabolite[a]	5.27	0.77	**238**
Fenfluramine	5.28	0.77	**264;** 270
2-Amino-5-nitrobenzophenone[a]	5.29	0.78	234; **361**
Metipranolol	5.30	0.78	**281**
Triprolidine	5.34	0.79	**231;** 281
Naproxene	5.34	0.79	**230;** 264; 272; 331
Amylocaine	5.34	0.79	**231;** 274
Buprenorphine	5.36	0.80	**288**
Verapamil[a]	5.38	0.81	**230;** 279
Buspirone	5.39	0.81	**236;** 300
Tianeptine	5.40	0.81	**269**
Midazolam	5.43	0.82	n.p.
Bupivacaine	5.43	0.82	**264**
Fenbufene	5.44	0.83	**285**
Carbinoxamine	5.44	0.83	**226;** 261
Ethyl loflazepate	5.45	0.83	**230;** 316
Pipotiazine[a]	5.50	0.85	235; **264;** 313
Zipeprol[a]	5.51	0.85	**259**
Loprazolam[a]	5.53	0.86	236; **329**
Amineptine	5.58	0.87	**266**
Cetirizine	5.59	0.88	**230**
Dexchlorpheniramine[a]	5.60	0.88	**225;** 262
Niflumic acid metabolite[a]	5.62	0.89	**294;** 365
Moperone	5.62	0.89	**247**
Oxaflozane	5.65	0.90	**262;** 269
Glafenine	5.67	0.90	**225;** 346
Cibenzoline	5.69	0.91	**222**
Medifoxamine[a]	5.71	0.92	**266;** 273
Astemizole	5.73	0.92	**278;** 283
Vinblastine	5.75	0.93	**268;** 310
Nicardipine	5.83	0.96	**238;** 255
Bisoprolol[a]	5.84	0.96	**225;** 272; 282
Moxisylyte	5.89	0.98	**275**
Diltiazem[a]	5.90	0.98	**238**

TABLE 1—*Continued.*

Compound	RT (min)	k′	λ_{max}(nm)
Glibornuride	5.92	0.99	**229;** 264; 274
Reserpine	5.94	0.99	**268;** 290
Propericiazine	5.94	0.99	233; **269**
Aconitine	5.95	1.00	**233;** 274
Alpidem metabolite 2[a]	5.96	1.00	**248;** 317
Nitrendipine	6.00	1.01	**236;** 355
Benzene	6.00	1.01	239; 244; **249;** 255; 262
Diazepam[a]	6.01	1.02	**229;** 316
Mianserine	6.02	1.02	**279**
Ramipril	6.04	1.03	**259**
2,4-Dichlorophenol[a]	6.06	1.03	**227;** 287
Haloperidol[a]	6.08	1.04	**221;** 247
Isothipendyl	6.09	1.04	**248;** 314
Oxametacine	6.12	1.05	**253;** 320
Hexamethylamine	6.15	1.06	**230**
Tetracaine	6.16	1.07	227; **313**
Alprenolol	6.18	1.07	**270;** 278
Buflomedil[a]	6.18	1.07	**278**
Aceprometazine	6.20	1.08	**242;** 275; 364
Glibenclamide	6.25	1.10	**229;** 301
Chlorophacinone[a]	6.30	1.11	**225;** 286; 313; 326
Doxepine[a]	6.31	1.12	**296**
Nimodipine	6.36	1.13	**238;** 357
Alpidem metabolite 3[a]	6.38	1.14	**247;** 314
Diphenhydramine[a]	6.39	1.14	**259**
Cyclizine	6.49	1.18	**225;** 260
Histapyrrodine	6.50	1.18	**249;** 296
Phenylbutazone	6.51	1.18	**239**
Demexiptiline	6.52	1.19	**230;** 294
Clozapine	6.53	1.19	**259;** 294
Proguanil	6.55	1.20	231; **259**
Trifluperidol	6.61	1.22	**247**
Medazepam	6.62	1.22	**255**
Cyamemazine[a]	6.71	1.25	231; **269**
Bumadizone	6.72	1.26	**235**
Suriclone	6.74	1.26	**244;** 322; 353
Propranolol[a]	6.76	1.27	**290;** 320
Acepromazine[a]	6.77	1.27	**243;** 278; 376
Dosulepine[a]	6.82	1.29	**230;** 305
Dextromoramide[a]	7.05	1.37	**260**
Fenoprofene	7.20	1.42	**272**
Dextropropoxyphene[a]	7.24	1.43	**259**
Loxapine[a]	7.37	1.47	**299**
Betaxolol[a]	7.38	1.48	**223;** 275
Propafenone[a]	7.38	1.48	**248;** 305
Promethazine[a]	7.46	1.50	**251;** 301
Thioproperazine	7.47	1.51	235; **266;** 317
Methadone	7.53	1.53	**261;** 269
Amoxapine[a]	7.57	1.54	**299**
Quinupramine	7.62	1.56	**248;** 298
Opipramol	7.62	1.56	**256;** 335
2-Amino-2′,5′-dichlorobenzophenone	7.67	1.57	**234;** 262; 394
Bepridil metabolite[a]	7.67	1.57	**242;** 291
Cyproheptadine	7.68	1.58	**225;** 286
Brompheniramine[a]	7.78	1.61	**226;** 262
Mefenidramine	7.95	1.67	n.p.
Protriptyline	7.97	1.67	**292**
Flurbiprofene	8.01	1.69	**247**
Tetrazepam	8.08	1.71	**227;** 307
Zorubicine	8.10	1.72	**234;** 255
2-Amino-5-chlorobenzophenone[a]	8.11	1.72	**236;** 391
Prazepam[a]	8.29	1.78	**229;** 313
Alimemazine[a]	8.35	1.80	**255;** 303
Loperamide	8.50	1.85	**260**
Imipramine[a]	8.53	1.86	**251**
Desipramine[a]	8.58	1.88	**251**
Levomepromazine	8.59	1.88	**252;** 305

TABLE 1—*Continued.*

Compound	RT (min)	k′	λ_{max}(nm)
Hydroxyzine[a]	8.62	1.89	**230**
Niflumic acid[a]	8.63	1.90	**288;** 335
Levopenbutolol	8.65	1.90	**270;** 277
Fluvoxamine[a]	8.71	1.92	**253**
Pimozide	8.77	1.94	**281**
Daunorubicine	8.90	1.99	**234;** 253; 291
Indometacine[a]	8.91	1.99	**253;** 320
Maprotiline	8.93	2.00	265; **272**
Tropatepine[a]	8.96	2.01	**231;** 259; 303
Etodolac	9.13	2.06	223; **273**
Fluoxetine[a]	9.14	2.07	**226;** 265; 275
Amitriptyline[a]	9.23	2.10	**240**
Nortriptyline[a]	9.28	2.11	**239**
Tioclomarol	9.41	2.16	288; **312**
Diclofenac[a]	9.51	2.19	**275**
Mefloquine	9.58	2.21	**222;** 282; 305; 316
Trimipramine	9.78	2.28	**251**
Chlorambucil	10.00	2.36	**259;** 303
Lidoflazine	10.35	2.47	**265;** 272
Ibuprofene[a]	10.58	2.55	**220;** 264
Floctafenine	10.73	2.60	**353**
Alpidem[a]	10.97	2.68	**247;** 317
Loratadine	11.29	2.79	**247**
Chlorpromazine[a]	11.50	2.86	**256;** 309
Clomipramine[a]	12.00	3.03	**252**
Carpipramine	12.58	3.22	**251**
Thioridazine	13.43	3.51	230; **264;** 313
Fentiazac	13.49	3.53	223; **247;** 300
Clemastine	14.48	3.86	**259**
Terfenadine metabolite[a]	14.78	3.96	**259**
5-Chloro-2-methylaminobenzophenone	15.50	4.20	**236**
Mefenamic Acid	16.48	4.53	**220;** 279; 352
Fluphenazine	16.88	4.66	**260;** 312
Prochlorperazine	18.25	5.12	**257;** 311
Penfluridol	18.25	5.12	**266;** 272
Bepridil[a]	18.30	5.14	**249;** 296
Terfenadine[a]	18.33	5.15	**259**
Trifluoperazine[a]	19.78	5.64	**260;** 312

For each compound are given: retention time (RT) in min; capacity factor (k; ′); UV absorption peaks (at pH = 2.6) between 220 and 400 nm (main peak in **bold type**).

[a] = compound identified at least one time in biological samples; n.p. = no marked peak between 220 and 400 nm. Compounds are listed according to their elution order.

performed on the same apparatus. The coefficients of variation of the RTs ranged from 1.02 % (prazepam at 1.0 μg/mL) to 5.37% (clomipramine at 1.0 μg/mL).

For each compound of the library, absolute recovery was measured at least one time by extracting and assaying drug-free haemolized whole blood samples spiked with the drugs tested at the concentration of 1.0 μg/mL, then by comparing the peak areas of these extracted standards with those of methanolic standards at the same concentration. For benzodiazepines, barbiturates, antidepressants, neuroleptics, beta-blockers and opiates, assays were carried out in multiplicate at various concentrations. Recoveries were found to be higher than 60% in 80% of the library's entries, and higher than 20% for all of them.

The lower limits of detectability were determined by extracting and assaying whole blood samples spiked with decreasing concentrations of the drugs tested, until a signal equivalent to three times the background noise was obtained; for this measurement, each drug was monitored at its wavelength of maximal absorbance (λ_{max}). The results were in the range 9 to 87 ng/mL for 27 neurolep-

tics [1]; 2.5 to 15 ng/mL for 25 antidepressants [2]; 8.5 to 54 ng/mL for 26 benzodiazepines; 19 to 34 ng/mL for 4 cyclopyrrolones and imidazopyridines [3]; 28 to 113 ng/mL for 18 beta-blockers; 5 to 93 ng/mL for 24 non-steroidal anti-inflammatory drugs (NSAIDs); 36 ng/mL and 42 ng/mL for morphine and codeine, respectively.

Owing to the diode-array detection, identification errors (that is, confusion between 2 compounds) require coincidence of both RTs and UV spectra. In order to estimate the specificity of our method, we have listed for each drug of the library the possible interfering compounds, characterized by their elution in the time window of the drug of interest (± 0.5 min for RT ≤ 9 min; ±1.0 min for RT > 9 min) and a spectral similarity above the cut-off chosen for identification (900/1000); results are summarized in Table 2.

Discussion

Due to its high separation power, universality (including thermolabile and non-volatile compounds), and ease of sample preparation, HPLC appears as a potentially very valuable method for STA, and has been proposed for this application since the 1970s [4,5]. However, at first it did not receive much attention, since the lack of reproducible column material led to poor intra- and inter-laboratory reproducibility of RTs, thus to low specificity when using single-wavelength UV detection [6–10].

TABLE 2—*Specificity study: list of compounds exhibiting similarity in both retention times and uv spectra.*

Compounds	Retention times (min)	Match (/1000)
7-aminoflunitrazepam/7-aminonitrazepam	3.16/3.18	935
Morphine/Codeine	3.32/3.44	911
Morphine/Nalorphine	3.32/3.52	978
Morphine/Codethyline	3.32/3.65	946
Codeine/6-monoacetylmorphine	3.44/3.48	901
Codeine/Codethyline	3.44/3.65	922
6-monoacetylmorphine/Codethyline	3.48/3.65	900
Lisinopril/Hydroxydebrisoquine	3.50/3.62	921
Lisinopril/Gluthetimide	3.50/3.83	934
Nalorphine/Codethyline	3.52/3.65	930
Hydroxydebrisoquine/Gluthetimide	3.62/3.83	939
Hydroxydebrisoquine/Ephedrine	3.62/3.95	934
Hydroxydebrisoquine/Debrisoquine	3.62/4.07	974
Gluthetimide/Ephedrine	3.83/3.95	900
Cluthetimide/Debrisoquine	3.83/4.07	931
Desmethylclobazam/Clobazam	3.85/4.00	946
Triazolam/Alprazolam	3.88/4.17	914
Estazolam/Alprazolam	3.94/4.17	972
Ephedrine/Debrisoquine	3.95/4.07	952
Ephedrine/Mephentermine	3.95/4.30	983
Dextromethorphane/Pentazocine	4.03/4.33	956
Debrisoquine/Mephentermine	4.07/4.30	960
Quinine/Quinidine	4.28/4.34	987
Quinine/Hydroquinidine	4.28/4.55	990
Lidocaine/Mepivacaine	4.45/4.54	973
Benperidol/Droperidol	4.62/4.65	920
Hydroxychloroquine/Chloroquine	4.65/4.72	998
Clorazepate/Ethyl loflazepate	5.20/5.45	958
Carbinoxamine/Dexchlorpheniramine	5.44/5.60	955
Amineptine/Ramipril	5.58/6.04	928
Loxapine/Amoxapine	7.37/7.57	981
Imipramine/Desipramine	8.53/8.58	993
Amitriptyline/Nortriptyline	9.23/9.28	985
Clomipramine/Carpipramine	12.00/12.58	921
Clemastine/Terfenadine metabolite	14.48/14.78	906

The identification of solutes from their retention behaviour in combination with their UV spectral features, due to the introduction of UV multichannel detectors (and more recently photodiode array detectors), considerably improved the specificity of HPLC and afforded it a number of the advantages previously enjoyed solely by GC/MS [11–17]. As shown by many applications reported throughout the 1980's, HPLC/DAD has rapidly proven itself as a screening tool in the field of single-class drug identification [18–28], as well as general-unknown analysis of biological fluids [29–39] (Table 3).

Similar to many authors reporting STA methods [29,32,34–39], we performed liquid/liquid extraction (LLE) rather than solid-phase extraction (SPE) for reasons of speed, lower cost and easier optimization. Chloroform/2-propanol/*n*-heptane (60:14:26, v/v/v), which has been initially employed for extraction of opiates and synthetic derivatives [40,41], was chosen as the extraction solvent since it is less prone to form emulsions, gives extracts that are clean enough for using a portion of them for eventual, subsequent GC/MS confirmation, and was found to provide good to excellent recoveries for a great variety of pharmaceuticals and drugs of abuse, which is of prime necessity for broadspectrum screening purposes. However, due to extraction under alkaline conditions (pH = 9.5), poor recoveries of 20 to 50 % were found for acidic compounds such as barbiturates, salicylates or some NSAIDs. This was not considered to be a severe drawback, since 1) these drugs only account for a small part of the pharmacopoeia, 2) most of them are usually given at large doses (hundreds mg/d or more) resulting in high plasma levels.

The day-to-day variability of retention times (1.02 to 5.37%) was comparable to that reported by other authors dealing with HPLC/DAD screening [42]. This points out the inadequacy of sole HPLC retention parameters, even when standardized as relative retention times [9] or retention indices [6–8,35,43–47], as an identification criterion under screening conditions.

A frequent criticism of HPLC/DAD is its poor sensitivity, especially when compared to classical single-wavelength spectrophotometers [11,15]. In practice our detection limits were found to be sufficient to allow identification of most of the library's compounds, at least at therapeutic blood levels, *a fortiori* in poisoning cases; however, false negatives may occur with some substances exhibiting activity and toxicity at low blood concentrations (for example, plant alkaloids like colchicine, triazolobenzodiazepines, some opiate synthetic derivatives, . . .).

The specificity of the method was judged excellent, with only 35 pairs of drugs exposed to the risk of misinterpretation of analytical data (Table 2). In fact, the only real problems may arise with the group of opiates (morphine, 6-monoacetylmorphine, codeine, codethyline) and nalorphine, which elute within a narrow time window (3.32 to 3.65 min) and exhibit very similar UV spectra. These difficulties become only theoretical if, as a guideline, every analytical report suggesting presence of drugs of abuse in biological samples (whatever the method employed) must systematically be confirmed by a GC/MS determination. In unfrequent situations where an absolute specificity is required with thermolabile and/or non-volatile compounds difficult to assay by GC/MS even when derivatized (for example, "big" alkaloids), LC/MS may represent an appropriate alternative by combining the universality of the liquid separation and the ultraspecific mass identification. Such LC/MS confirmation methods are presently under development in our laboratory and will be presented in further reports.

The entire screening procedure (including single-step extraction, evaporation, and 20 min chromatographic run) may be achieved

TABLE 3—*HPLC/DAD methods for toxicological screening: review 1986–1993.*

Author (year) [reference]	Column	Mobile Phase	Elution (flow)	Library
Cosbey (1986) [29]	Hypersil ODS, 5 μm (160 × 4.5 mm, i.d.)	MeOH/H_3PO_4 + 7S.Ac, pH 2.1 (60:40, v/v)	ISOCRATIC (2.0 ml/min)	62 pharmaceuticals and drugs of abuse in blood and urine
Minder *et al.* (1987) [30,31]	Supelcosil 18DB, 5 μm (250 × 4.6 mm, i.d.)	ACN/MeOH/0.1 M phosphate buffer, pH 2.7 (44:6:50, v/v/v) + 0.02% TEA	ISOCRATIC (1.0 ml/mm)	4B pharmaceuticals (BZD, neuroleptics, antidepressants) (methanolic standards)
Mura *et al.* (1987) [18]	μBondapak 5 μm, reversed-phase (300 × 4.6 mm, i.d.)	ACN/phosphate buffer, pH 5.4	GRADIENT ACN 38% (15 min) ACN 38% → 70% (7 min) ACN 70% (18 min) (0.7 ml/min)	21 BZD + metabolites
Hill and Langner (1987) [13]	1) (*acidic compounds*) Zorbax C8 2) (*basic compounds*) PRP-1	1) A = 0.1% H_3PO_4 B = ACN/H_2O/H_3PO_4 (89.9:10:0.1, v/v/v) 2) A = 1.0% NH_4OH B = ACN/NH_4OH (99:1, v/v)	1) GRADIENT B: 0% → 100% (30 min) (2.0 ml/min) 2) GRADIENT B: 0% → 100% (30 min) (2.0 ml/min)	1) 157 pharmaceuticals (*acidic system*) 2) 144 pharmaceuticals (*basic system*) (methanolic standards)
Fullinfaw *et al.* (1987) [20]	LiChrosorb RP-18, 5 μm (125 × 4.0 mm, i.d.)	ACN/10 mM phosphate buffer, pH 3.0	GRADIENT ACN 10% (1.5 min) ACN 10% → 35% (2 min)	12 diuretics in urine
Li and Zhang (1987) [19]	GYQG-C18, 5 μm (150 × 2.1 mm, i.d.)	MeOH/2% CH_3COOH (90:10, v/v)	ISOCRATIC (0.5 ml/min)	3 β-blockers (pindolol, oxprenolol, propranolol) in urine
Minder *et al.* (1988) [23]	Supelcosil 18DB, 5 μm (250 × 4.6 mm, i.d.)	ACN/MeOH/50 mM KH_2PO_4 pH 2.6 (40:8:52, v/v/v) + 1.43 μM TEA *then (after 1st cell.)* +0.1 M $(NH_4)_2HPO_4$, pH 10.5 (0.1 ml/min)	ISOCRATIC (0.5 ml/min—10 min; 1.0 ml/min—10 min; 2.0 ml/min—5 min) (detection in 2 successive flow cells, with on-line alkalinization of mobile phase)	17 barbiturates in serum
Fullinfaw *et al.* (1988) [21]	Hypersil ODS, 5 μm (100 × 2.0 mm, i.d.)	ACN /10 mM phosphate buffer, pH 5.0	GRADIENT ACN 20% → 45% (3 min) (0.5 ml/min)	7 laxatives in urine
Snoeren *et al.* (1988) [32]	LiChrospher 100 RP-18, 5 μm (125 × 4.0 mm, i.d.)	ACN/phosphate buffer, pH 3.3 (47:53, v/v) + 0.0146% TEA	ISOCRATIC (0.6 ml/min)	221 pharmaceuticals and drugs of abuse in biological fluids
Verstraete and Wieme (1988) [22]	Chrompack Chromspher C18	McOH/ACN /0.1 M phosphate buffer, pH 2.7/TEA (3:37:60:0.02, v/v/v/v)	ISOCRATIC (0.5 ml/min)	24 BZD in serum
Minder *et al.* (1989) [24]	Supelcosil 18DB, 5μm (250 × 4.6 mm, i.d.)	MeOH/H_2O (70:30, v/v)	ISOCRATIC	13 benzophenones in urine
Cooper *et al.* (1989) [25]	1) Hypersil ODS, 5 μm (200 × 4.6 mm, i.d.) 2) LiChrosorb RP-18, 5μm (200 × 4.6 mm, i.d.)	ACN /50 mM phosphate buffer, pH 3.0	GRADIENT ACN 15% → 80% (18 min) (1.0 ml/min)	23 diuretics in urine
Park *et al.* (1990) [26]	Hypersil ODS, 5 μm (100 × 4.6 mm, i.d.)	ACN/phosphate buffer, pH 6.8	GRADIENT ACN 4% → 30% (10 min) ACN 30% → 45% (5 min) ACN 45% → 60% (3 min) (1.0 ml/min)	13 diuretics in urine
Logan *et al.* (1990) [33]	Hibar LiChrospher 100 CH-8/11 (C8) (250 × 4.6 mm, i.d.)	ACN/50 mM phosphate buffer, pH 3.2	GRADIENT ACN 10% → 50% (15 min) (1.5 ml/min)	100 basic pharmaceuticals and drugs of abuse in urine
Martens *et al.* (1990) [34]	LiChrospher 100 RP-18, 5 μm	ACN/phosphate buffer, pH 4.2 (47:53, v/v) + 0.0146% TEA	ISOCRATIC	≈250 pharmaceuticals and drugs of abuse in serum

TABLE 3—*Continued.*

Author (year) [reference]	Column	Mobile Phase	Elution (flow)	Library
Bogusz *et al.* (1991) [*35*]	Superspher 10 RP-18, 4 μm (125 × 4.0 mm, i.d.)	ACN/25 mM phosphate buffer, pH 3.0	GRADIENT ACN 0% → 70% (30 min) ACN 70% (5 min) (1.0 ml/min)	225 pharmaceuticals, toxicants and drugs of abuse in post-mortem blood
Felscher (1991) [*36*]	LiChrospher RP-18, 5 μm (100 × 2.1 mm, i.d.)	1) ACN/phosphate buffer, pH 2.3 (31.2:68.8, w/w) 2) MeOH/H_2O (60:40, v/v) 3) ACN/10 mM H_2SO_4 + 9 mM 7S.Ac, pH 1.7 (85:15, v/v)	ISOCRATIC (0.5 ml/min)	≈250 pharmaceuticals and drugs of abuse in biological fluids
Felscher (1991) [*37*]	LiChrospher RP-18, 5 μm (100 × 2.1 mm, i.d.)	1′) = 1) (see above) 2′) = 1′/phosphate buffer, pH 2.3 (40:60, v/v) 3′) = 1′/ACN (92:8, v/v)	ISOCRATIC (0.5 ml/min)	≈250 pharmaceuticals and drugs of abuse in biological fluids
Tsai *et al.* (1991) [*27*]	Bondclone ODS, 10 μm (300 × 3.9 mm, i.d.)	ACN/50 mM NaH_2PO_4 + 1.59 g/l PPA, pH 3.0	GRADIENT ACN 15% (1.5 min) ACN 15% → 45% (5.5 min) ACN 45% → 59% (5 min) ACN 59% → 80% (9 min) (1.0 ml/min)	14 diuretics in urine
Koves and Wells (1992) [*38*]	1) Apex ODS, 5 μm (250 × 4.6 mm, i.d.) 2) Waters μPhenyl, 5μm (150 × 3.9 mm, i.d.)	1) ACN/0.025% H_3PO_4/TEA (25:10:5, v/v/v), pH 3.4 2) ACN/0.025% H_3PO_4 (50:50, v/v)	1) ISOCRATIC (0.8 ml/min) 2) ISOCRATIC (0.6 ml/min)	119 basic pharmaceuticals and drugs of abuse in postmortem blood
Mußhoff and Daldrup (1992) [*28*]	Kontrosorb 10 RP-18 (250 × 4.6 mm, i.d.)	ACN/6.66 g/l KH_2PO_4, pH 2.3 (156:340, w/w)	ISOCRATIC (1.3 ml/min)	8 BZD in serum and postmortem blood
Kohn (1993) [*39*]	Hypersil ODS, 5 μm (100 × 2.1 mm, i.d.)	ACN/20 mM KH_2PO_4, pH 6.0 + 0.05% TEA	GRADIENT ACN 15% → 40% (10 min) ACN 40% → 75% (3.5 min) ACN 75% → 80% (2.5 min) ACN 80% (2 min) (0.4 ml/min)	376 pharmaceuticals in plasma or blood

NOTE: Abbreviations: 7S.Ac: heptanesulfonic acid; ACN: acetonitrile; BZD: benzodiazepines; MeOH: methanol; TEA: triethylamine.

in less than 120 min, making our method convenient not only for forensic investigations, but also for emergency-room situations. The equipment costs lay in the same range that GC/MS (about $40,000 to 50,000 for a complete apparatus with autosampler, quaternary pump, DAD and computer), but in practice our method was found to be particularly economical due to 1) the robustness of HPLC equipment leading to low maintenance costs (especially in comparison to GC/MS, which is at least 3 times more expensive for a similar use), 2) the choice of isocratic elution allowing mobile phase recycling, which results in a dramatically reduced solvent consumption.

Immunoassays remain as irreplaceable tools for initial STA in forensic situations. However it seems more and more obvious that they are not sufficient to ensure a comprehensive and unequivocal screening, since 1) they generally provide only a class determination (for example, opiates, barbiturates, tricyclic antidepressants, . . .); 2) they are prone to interferences resulting from cross-reactivity between structurally related compounds (tricyclic antidepressants/phenothiazines, amphetamine derivatives/putrefactive bases [*48,49*], . . .). As shown in some previous reports, our screening procedure appears to be of particular value for investigation of poisonings requiring rapid and nominal drug characterization, or involving compounds not identified by immunoassays, such as neuroleptics [*1,50,51*], non-imipraminic antidepressants [*2,52*], synthetic opiate derivatives or antagonists [*53,54*], "benzodiazepine-like" hypnotics and sedatives (cyclopyrrolones, imidazopyridines) [*3,51,55*], beta-blockers [*56,57*] or other drugs [*58,59*].

Conclusion

This method is simple, rapid, highly specific and sensitive enough for poisoning cases. Although primarily devoted to qualitative determinations, it may be easily adapted to quantitative measurements [*58,59*]. In addition, as new compounds are encountered they may easily be added to the library. In conclusion, it is likely that HPLC/DAD procedures will take an increasingly important place among drug screening strategies in forensic situations.

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