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Use of high-performance liquid chromatography with photodiode-array UV detection for the creation of a 600-compound library Application to forensic toxicology

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Abstract

A high-performance liquid chromatographic method is described with photodiode array detection for systematic toxicological analysis in human blood and urine. After a single step liquid–liquid extraction using Toxi-Tube A, drugs are analyzed with a multi step gradient (phosphate pH 3.8–acetonitrile) on a Symmetry C_8 5-μm column (250 mm×4.6 mm I.D.) (Waters), operated at 30°C. The flow-rate is varied during the run from 1 ml/min to 1.5 ml/min. Full UV spectra are recorded on-line during the 28 min chromatographic run. Enhanced performances for drug detection are obtained with this method due to (a) reduction of peak-tailing for basic drugs, (b) lower identification limits, (c) more stable retention times and (d) larger number of referenced drugs (684 spectrum registered). Application of real samples has been demonstrated.

Keywords: Forensic analysis; Toxicological screening; Retention times; Drugs

1. Introduction

The introduction of photodiode array (PDA) detection in the early 1980s opened a new area for HPLC users. This technology, now available from more than 10 manufacturers, is of common use in every toxicological laboratory and is also widely used in the pharmaceutical industry for peak homogeneity determination. Indeed, for toxicological purposes, the main use is for peak identification. Numerous procedures have been proposed for systematic toxicological analysis (STA), including colorimetric determinations, ultraviolet (UV) spectrophotometry, immunoassays, thin-layer chromatography, gas chromatography coupled to classical or mass spectrometric (GC–MS) detectors as well as normal or reversed-phase HPLC. HPLC presents the advantage of allowing identification of thermally unstable compounds which cannot be analyzed by gas chromatography under normal conditions. Thus, GC–MS and HPLC–PDA technologies represent two different approaches, to identify and quantify the widest possible number of pharmacological classes of drugs. These techniques are complementary and additive.

The power of a method depends on both its ability to separate the analytes in the presence of a large number of components and its capacity for peak identification by library matching. As an illustration of such affirmations, this work describes an improvement in the step separation methods previously reported [1–15]), as well as an increased number of referenced substances. Applicability to forensic toxicology will be demonstrated.

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2. Experimental

2.1. Instrumentation

HPLC equipment consists of a quaternary gradient pump Waters 600 (Saint Quentin en Yvelines, France), an autoinjector Waters 717 Plus and a PDA UV detector Waters 996. System control, data acquisition and process are made by The Waters Millennium software. The separation column is a Waters Symmetry C_8, 250 mm×4.6 mm I.D. (5-μm particle size) equipped with a 20-mm guard column (Waters Symmetry C_{18}). Separation conditions are as follows: column temperature 30°C, solvent A= phosphate buffer (pH=3.8), solvent B=acetonitrile; step gradient is 15% B for 6.5 min, then 35% until 25 min, then 80% B for 3 min. Total chromatography duration is 28 min. The equilibration time between two consecutive samples was set at 7 min. The flow-rate of the mobile phase was 1 ml/min for 6.5 min, then linear increase to 1.5 ml/min from 6.5 min to 25 min and hold for 3 min: re-equilibration is made at 1.5 ml/min. Injection volume was varied from 10 to 30 μl. UV spectra from 200 to 350 nm (resolution 1.2 nm) are recorded on-line during the chromatographic run. Solute identification may be automatically performed by comparison of analytical data (retention times and UV spectra), with the 684 UV spectra of pharmaceuticals, pesticides, toxicants and drugs of abuse stored in the users built library (see Table 1).

For the extraction of the biological fluids; i.e. urine and whole blood, we used Toxi-Tube A and Toxi-Tube B from Toxi-Lab (Irvine, CA, USA) supplied by Amilabo (Chassieu, France).

2.2. Reagents

Acetonitrile, orthophosphoric acid 10% and anhydrous monobasic sodium phosphate (NaH_2PO_4) of analytical grade were from Carlo Erba (Milano, Italy). Pharmaceutical standards were graciously offered by the different manufacturers.

Phosphate buffer 50 m*M*, pH=3.8 for the mobile phase was prepared by dissolving 6 g of NaH_2PO_4 in 1 l of distilled water and adjusting to pH=3.8 with orthophosphoric acid 10%. This pH 3.8 phosphate buffer has a low buffering capacity; however, it was judged adequate for the present method. Moreover, it has a very low UV absorption.

2.3. Extraction and injection

We have followed the general procedure recommended by the supplier. In a Toxi-Tube A we successively added 1 ml of urine or whole blood and 3 ml of distilled water. After mixing by gentle inversion for 5 min, the tube was centrifuged at 1500 *g* for 5 min. The organic phase was removed, evaporated to dryness at 40°C under a stream of nitrogen. Reconstitution was performed by adding 50 μl of acetonitrile–water (50:50, v/v) to the dry residue, then vortex mixed for 10 s. The extract was transferred to a microtube, Eppendorf type from ATGC (Paris, France), and centrifuged at 7500 *g* for 2 min. This important step gives a clear extract separated from oil residue and impurities. These residues are frequently observed when extracting a complex matrix like putrefied materials. The clear extract, obtained under these conditions, gives a low interference chromatographic analysis which will enhance the drug detection. For a blood extract, the whole clear phase is injected, while only 10 μl is used for urine analysis.

Toxi-Tube A was originally dedicated to the extraction of basic and neutral drugs. Due to the alkaline extraction conditions, poor recoveries ranging from 20 to 50% were observed for acidic compounds like barbiturates, salicylates or some diuretics, non-steroidal anti-inflammatory drugs (NSAIDs) or anticoagulants. For some compounds rather, it must be considered as an advantage. As a point of fact, barbiturates, salicylates and even NSAIDs or diuretics are usually administrated at large doses resulting in high plasma levels. Too high concentration may lead to detection saturation, which impacts identification; best results are obtained with less than 1 AU peaks. Components present in large amounts will be better identified if extracted with low recoveries. Indeed, for compounds like anticoagulants and some antibiotics, an extraction using Toxi-Tube B (for acidic compounds) would be preferred.

Table 1
Spectra listed in the library

Retention time (min)	Spectrum name	γ_{max}	Retention time (min)	Spectrum name	γ_{max}
2.597	Vitamin B_1	200.5	3.61	Niacinamide	214.6
2.613	Histidine	211.1	3.625	Diprophylline	206.4
2.687	Pholcodine	211.1	3.628	Acefylline heptaminol	207.5
2.697	Adenosine	206.4	3.635	Caffeine metabolite 1	206.4
2.74	Imidazole	205.2	3.637	Atenolol	200.5
2.772	4-Hydroxybutyrate	207.5	3.682	Theodrenaline	201.7
2.8	Norepinephrine	200.5	3.683	Terbutaline	200.5
2.803	Metformine	233.4	3.703	Crimidine	251.1
2.81	Dihydralazine	219.3	3.71	Amphetamine	200.5
2.835	Glutathion	200.5	3.74	Ranitidine	228.7
2.852	Germall 115	200.5	3.772	Vitamin B_5	200.5
2.863	Pralidoxime	295	3.777	Cyanocobalamine	207.5
2.87	Epinephrine	200.5	3.79	Theobromine	204
2.873	Moroxydine	236.9	3.795	Sulfaguanidine	200.5
2.895	Vitamin B_6	200.5	3.808	Cefatrizine	200.5
2.928	Vitamin C	249.9	3.823	Pyrazinamide	208.7
2.96	Methyldopa	200.5	3.827	Ampicilline	200.5
2.97	Acamprosate	200.5	3.842	Sotalol	200.5
2.985	Fumaric Acid	206.4	3.858	Sulpiride	212.2
2.988	Methionine	200.5	3.867	Bidesethylchloroquine	219.3
3.002	3-Hydroxytyramine	200.5	3.875	Cefalexine Peak 1	200.5
3.008	Pyroglutamic acid	200.5	4.15	Metaproterenol	200.5
3.02	Synephrine	200.5	4.172	Phloroglucinol	202.8
3.027	Pancuronium	200.5	4.185	4-Methyldopamine	200.5
3.052	Flucytosine	200.5	4.237	Tiapride metabolite	212.2
3.067	Amoxicilline	200.5	4.53	Lisuride	209.9
3.073	Aciclovir	200.5	4.622	Pilocarpine	214.6
3.1	Arecoline	207.5	4.637	Actinoquinol	202.8
3.11	Blood interference 1	202.8	4.675	Monodesethylchloroquine	219.3
3.112	Kathon GC	200.5	4.7	Cotinine	200.5
3.155	Betahistine	200.5	4.7	Dihydrocodeine	208.7
3.163	Nicotinic acid	209.9	4.737	5-Aminosalicylic acid	205.2
3.188	Cefadroxil	200.5	4.76	Nalorphine	211.1
3.227	Lactic acid	200.5	4.782	Neostigmine	200.5
3.228	Pyridostigmine	200.5	4.792	Cefalexine peak 2	200.5
3.23	3-Methyldopamine	200.5	4.823	Cefixime	287.8
3.233	Neopynamine	209.9	4.827	Caffeine metabolite 2	201.7
3.283	Cyromazine	214.6	4.877	Theophylline	202.8
3.295	Piracetam	200.5	4.975	Codeine	212.2
3.302	Nizatidine	316.4	4.993	Coumatetralyl	264.1
3.315	Morphine	211.1	5.015	Norephedrine	205.2
3.343	Psilocybine	220.5	5.03	Acetylmethionine	200.5
3.4	Mebezonium	225.2	5.047	Sulfamilamine	200.5
3.432	Enalapril	211.1	5.078	Colchicosine	200.5
3.433	Fluoro-uracile	204	5.218	Procaine	292.6
3.462	Pirisudanol	209.9	5.277	Esculine	202.8
3.487	Famotidine	202.8	5.32	Colchicoside	244
3.575	Levodopa	200.5	5.34	Ceftriaxone	245.2
3.578	Blood interference 13	211.1	5.442	Chloroquine	221.6
3.583	Folic acid	200.5	5.468	Tiapride	213.4
3.6	Dacarbazine	323.5	5.592	Paracetamol	200.5
3.602	Cimetidine	200.5	5.655	Ephedrine	206.4
3.608	Amiloride	214.6	5.875	Paramino salicylic acid	207.5

(*Continued on p. 152*)

Table 1 (*continued*)

Retention time (min)	Spectrum name	γ_{max}	Retention time (min)	Spectrum name	γ_{max}
5.907	Saccharine	200.5	9.397	Hydrochlorothiazine	226.3
5.91	Bamethane	200.5	9.492	Moclobemide metabolite 1	200.5
5.935	Carteolol	214.6	9.637	Ketamine	202.8
6.06	Trimetazidine	206.4	9.652	Captopril	200.5
6.087	Naltrexone	205.2	9.657	Hyoscyamine	200.5
6.128	Clonidine	200.5	9.668	Secnidazole	318.8
6.59	Phthalic acid	201.7	9.678	Benzoylecgonine	200.5
6.647	Caffeine	205.2	9.708	Moclobemide metabolite 2	200.5
6.77	Nadolol	200.5	9.76	Minoxidil	231.1
6.778	Metronidazole	320	9.8	Aspartame	200.5
6.927	Acetazolamide	265.3	9.8	Rilmenidine	200.5
6.97	Levamisole	213.4	9.825	Acetylleucine	200.5
6.97	Tetramisol	214.6	9.888	Tetracycline	274.8
7	Thiamphenicol	200.5	9.915	Metoclopramide	213.4
7.022	Diaveridine	200.5	9.922	Lidocaine	200.5
7.12	Amisulpride metabolite 1	226.3	9.942	Moclobemide metabolite 3	200.5
7.182	Vitamin B_2	267.7	9.955	Dimetridazole	317.6
7.243	Dropropizine	200.5	10	MDMA metabolite	200.5
7.32	6-Acetylmorphine	208.7	10.007	Molsidomine	312.8
7.39	Scopolamine	200.5	10.068	4-Chlorophenylbiguanide	200.5
7.672	Enoxacine	268.9	10.1	Rutine	204
7.688	Clomethiazole metabolite	1247.6	10.118	Dimetridozole	320
7.953	Metoclopramide metabolite	207.5	10.198	Cinchonine	202.8
8.007	Dimerazole	200.5	10.218	Moclobemide	200.5
8.027	Resorcino	200	10.233	Acebutolol	233.4
8.058	MDA	200.5	10.235	Cafedrine	206.4
8.068	Boldine	218.1	10.288	Bamifylline	207.5
8.253	Eserine	204	10.295	Timolol	296.1
8.265	Ronidazole	308	10.323	Nitrofural	260.6
8.282	Trimethoprime	205.2	10.37Z	Opiclone	200.5
8.37	Vinblastine	200.5	10.37	Alfuzosine	244
8.375	Sulfadiazine	200.5	10.388	Atropine	200.5
8.407	Blood interference 11	219.3	10.445	Barbital	200.5
8.423	Cefazoline	200.5	10.48C	Inchonidine	202.8
8.433	Methamphetamine	206.4	10.485	Ethosuximide	200.5
8.568	Pindolol	215.8	10.543	Ambemonium	200.5
8.648	Ofloxacine	295	10.563	Tinidazole	317.6
8.673	Lidocaine metabolite	200.5	10.582	Isoquercitrin	205.2
8.688	Amfepramone	200.5	10.608	Prazosine	246.4
8.705	Triamterene	215.8	10.623	Nicethamide	200.5
8.735	Codethyline	211.1	10.63	Clomethiazole metabolite	2247.6
8.89	Vitamin H	200.5	10.637	Rosmarinic acid	200.5
8.923	Amisulpride	225.2	10.712	Selegiline	207.5
8.927	Furaltadone	347.4	10.722	Metoprolol	200.5
8.942	Pefloxacine	278.3	10.763	Acepromazine	249.9
8.962	Apomorphine	206.4	10.793	Cycloguanil	200.5
8.963	Caffeic acid	321.2	10.795	Moclobemide metabolite 4	200.5
9.022	Sulfathiozol	200.5	10.802	Clenbuterol	211.1
9.058	MDMA	200.5	10.918	Pipamperone	200.5
9.102	Ciprofloxacine	278.3	10.937	Amisulpride metabolite 2	226.3
9.157	Noramidopyrine	200.5	10.993	Viloxazine	200.5
9.158	Viloxazine metabolite	200.5	11.025	Quinidine	208.7
9.202	Strychnine	207.5	11.047	Desoxy-2-phenobarbital	200.5
9.385	Emetine	202.8	11.092	Niaprazine	200.5

Table 1 (*continued*)

Retention time (min)	Spectrum name	γ_{max}	Retention time (min)	Spectrum name	γ_{max}
11.13	Primidone	200.5	12.497	Pyrimethamine	208.7
11.138	Carbimazole	200.5	12.522	Pentazocine	200.5
11.147	Doxylamine	200.5	12.523	Buspirone	236.9
11.152	Heroin	206.4	12.533	Indoramine	200.5
11.185	MDPA	200.5	12.583	Dapsone	200.5
11.217	Sulfamethoxypyridazine	200.5	12.583	Desethylatrazine	213.4
11.225	Minaprine	204	12.653	Nefopam	200.5
11.253	Quinine	208	12.683	Trazodone	211.1
11.3	Buflomedil	201.7	12.733	Tenoxicam	200.5
11.387	Guanfacine	200.5	12.758	Piperacilline	200.5
11.398	Fluconazole	200.5	12.768	Cyamemazine metabolite 2	272.4
11.435	Guaifenesine	200.5	12.81	Carbinoxamine	200.5
11.438	Blood interference 7	219.3	12.81	Chlorpheniramine metabolite	200.5
11.445	Disopyramide	200.5	12.827	Noscapine	213.4
11.468	Mexiletine	200.5	12.863	Trifluoperazine metabolite	2232.2
11.477	Pentoxifylline	206.4	12.913	Fenozolone	220.5
11.493	Celiprolol	232.2	12.923	Melatonine	200.5
11.493	Metacycline	242.9	12.925	Chlorpheniramine	200.5
11.503	Blood interference 16	268.9	12.93	Melfalan	201.7
11.51	Yohimbine	220.5	12.97	Sulbutiamine	200.5
11.6	Alimemazine metabolite 3	233.4	12.977	Cyamemazine metabolite 3	208.7
11.602	Promethazine metabolite 2	201.7	13.012	Sultopride	212.2
11.687	Vanilline	231.1	13.05	Alimemazine metabolite 2	253.5
11.707	Protopine	205.2	13.055	Fenfluramine	207.5
11.77	Pethidine	200.5	13.06	Propranolol	213.4
11.78	Acetiamin	200.5	13.118	Colchicine	244
11.795	Tiemonium	200.5	13.127	Dexamethasone	241.7
11.82	Amitriptyline metabolite	1206.4	13.133	Tripolidine	200.5
11.838	Chlorpromazine metabolite	3238.1	13.142	Estriol	200.5
11.853	Metamitron	200.5	13.143	Cibenzoline	200.5
11.882	Zolpidem	208.7	13.16	Astemizole	200.5
11.897	Mefenorex	207.5	13.197	Dipyridamole	285.5
11.9	Zipeprol metabolite	205.2	13.222	Difemerine	200.5
11.903	Pipamperone metabolite	200.5	13.27	Clidinium	200.5
11.92	Cocaine	200.5	13.277	β-methasone	200.5
11.962	Clindamycine	200.5	13.283	Alimemazine metabolite 1	202.8
11.997	Minaprine metabolite	202.8	13.312	Dextrometorphan	200.5
11.997	N-Acetyl-L-tyrosine ethyl ester	200.5	13.345	Sulfadoxine	200.5
12.002	Cortivazol	207.5	13.37	Blood interference 18	208.7
12.003	LSD	200.5	13.375	Phenoxy ethanol	200.5
12.003	Amitriptyline metabolite	2206.4	13.387	Loprazolam	200.5
12.017	Oxprenolol	200.5	13.393	Tobramycine	200.5
12.057	Doxorubicine	232.2	13.405	Betaxolol	200.5
12.08	Vincamine	221.6	13.422	Phenol	200.5
12.082	Netilmicine	206.4	13.43	Nifuroxazide	200.5
12.12	Salicylic acid	202.8	13.445	Sulfamethoxazole	200.5
12.12	Papaverine	251.1	13.45	Zipeprol	205.2
12.122	Alimemazine metabolite 5	226.3	13.467	Isothipendyl	248.8
12.232	N-Butylhyoscine	200.5	13.467	Rosoxacine	271.2
12.24	Furazolidone	347.4	13.48	Harpagoside	279.5
12.283	Bisoprolol	200.5	13.532	Chlorhexidine	200.5
12.293	Chloridazone	227.5	13.61	Proguanil	200.5
12.328	Blood interference 17	215.8	13.615	Phosdrin	215.8
12.355	Domperidone	207.5	13.615	Bentazone	224
12.445	Nadoxolol	211.1	13.637	Pimaricine	303.3

(*Continued on p. 154*)

Table 1 (*continued*)

Retention time (min)	Spectrum name	γ_{max}	Retention time (min)	Spectrum name	γ_{max}
13.647	Veratrine	220.5	14.655	EDDP	200.5
13.69	Tetracaine	312.8	14.66	Promethazine metabolite 1	200.5
13.698	Perindopril	206.4	14.687	Bacampicilline	200.5
13.737	Tianeptine metabolite	207.5	14.695	Pipothiazine	262.9
13.765	Vincristine	220.5	14.732	Bromazepam	232.2
13.787	Mianserine	200.5	14.735	Sulfadimethoxine	200.5
13.787	Gaiacol	200.5	14.76	Oxacilline	200.5
13.808	Cicletanine	200.5	14.763	Sulfachloropyrazine	271.2
13.813	Ticlopidine	200.5	14.847	Biperidene	200.5
13.825	Naproxen metabolite 1	231.1	14.858	Butobarbital	200.5
13.833	Adrafinil	200.5	14.87	Desipramine	200.5
13.833	Medifoxamine	200.5	14.873	Midazolam	200.5
13.852	Digoxine	220.5	14.877	Tianeptine	206.4
13.868	Terpine	312.8	14.907	Trichloromethiazide	225.2
13.912	Clobenzorex	200.5	14.91	Cyamemazine metabolite 1	270
13.935	Brompheniramine	200.5	14.943	Dosulepine	200.5
13.992	Diltiazem	200.5	14.955	Benzydamine	215.8
13.993	Phenobarbital	200.5	14.985	Nemonapride	212.2
14.028	Naloxone	200.5	14.993	Cyamemazine	270
14.032	Sisomicine	200.5	15.015	Cyproheptadine	224
14.032	Amineptine	200.5	15.04	Dextromoramide metabolite	200.5
14.035	Buprenorphine	212.2	15.093	Amlodipine	200.5
14.037	Gentamicine	200.5	15.113	Imipramine	200.5
14.04	Methylparaben	200.5	15.128	Propafenone	211.1
14.04	Chlorpromazine metabolite	4204	15.143	Aldicarb	200.5
14.053	Dibekacine	200.5	15.168	Quinupramine	200.5
14.065	Omeprazole	200.5	15.17	Furosemide	234.6
14.092	Dipheniramine	200.5	15.197	Pizotifene	200.5
14.095	Doxepine	206.4	15.212	Thioproperazine	265.3
14.105	Chloramphenicol	200.5	15.223	Chlordiazepoxide	244
14.107	Toloxatone	204	15.257	Alimemazine	253.5
14.113	Prednisolone	246.4	15.262	2,4-D	200.5
14.122	Phenothiazine ring	202.8	15.267	Hydroxyzine	200.5
14.157	Mianserine metabolite	200.5	15.275	Paroxetine	200.5
14.163	Opipramol	255.8	15.298	Trihexyphenidyle	200.5
14.168	Quercetine	202.8	15.3	Levomepromazine metabolite	1212.2
14.168	Propericyazine	270	15.302	Alimemazine metabolite 4	200.5
14.178	Prednisone	241.7	15.343	Dosulepine metabolite	200.5
14.187	Amoxapine	211.1	15.347	Fluvoxamine	200.5
14.202	Fentanyl	255.8	15.36	Methylclothiazide	226.3
14.225	Aceprometazine	240.5	15.365	Verapamil	201.7
14.265	Phenothiazine ring 2	204	15.425	Tropatepine	200.5
14.35	Naproxen metabolite 2	231.1	15.452	Nicergoline	202.8
14.352	Toloxatone metabolite	205.2	15.478	Norpropoxyphene	200.5
14.367	Cilazapril	200.5	15.493	Chlormezanone	200.5
14.415	Haloperidol	200.5	15.5	Parconazole	202.8
14.417	Flecaine	204	15.508	Maprotiline	200.5
14.475	Carbutamine	200.5	15.528	Nicardipine	205.2
14.482	Promethazine	251.1	15.57	Fenoverine	200.5
14.547	Carbutamide	200.5	15.582	Pentoxyverine	200.5
14.553	Loxapine	209.9	15.585	Proscillaridine A	200.5
14.588	Trimebutine	213.4	15.603	Nortriptyline	206.4
14.608	Naproxen metabolite 3	232.2	15.622	Prifinium	200.5
14.625	Lobeline	200.5	15.625	Levomepromazine metabolite	2251.1
14.627	Cisapride	214.6	15.645	Phenytoin metabolite	207.5

Table 1 (*continued*)

Retention time (min)	Spectrum name	γ_{max}	Retention time (min)	Spectrum name	γ_{max}
15.678	Ramipril	206.4	16.57	Piroxicam	200.5
15.683	Cetirizine	200.5	16.583	Vinylbital	200.5
15.702	Cloxacilline	200.5	16.597	Mefloquine	222.8
15.718	Amphotericine B	346.2	16.613	Lanzoprazole	200.5
15.72	Blood interference 19	207.5	16.617	Norclomipramine	200.5
15.73	Chlorpromazine metabolite	1254.7	16.617	Amobarbital	200.5
15.738	Ketoconazole	202.8	16.627	Sulindac	200.5
15.752	Simazine	220.5	16.652	Bromocriptine	200.5
15.753	Methadone	200.5	16.745	Flubendazole	211.1
15.763	Carbamazepine	213.4	16.745	Oxazepam	228.7
15.797	Oxatomide	205.2	16.763	Josamycine	231.1
15.813	Pivampicilline	200.5	16.782	Quinapril	200.5
15.82	Dextropropoxyphene	200.5	16.882	Bromazepam metabolite	200.5
15.83	Medazepam	200.5	16.927	Nitrazepam	200.5
15.832	Naftidrofuryl	225.2	16.947	Dichlorprop	200.5
15.833	Roxithromycine	200.5	16.967	Aprindine	200.5
15.835	Dextromoramide	200.5	16.967	Benzophenone	200.5
15.842	Levomepromazine	251.1	16.972	Alprazolam	220.5
15.845	2,4-Dinitrophenyl hydrazine	347.4	16.993	Trandolapril	206.4
15.865	Dextromoramide active form	200.5	17.003	Benazepril	205.2
15.877	MCPA	200.5	17.04	Dimethyl phtalate	200.5
15.878	Amitriptyline	206.4	17.11	Clobazam metabolite 1	228.7
15.928	Levopenbutolol	200.5	17.168	Thioridazine	262.9
15.943	Trimipramine	200.5	17.175	Lorazepam	228.7
15.958	Clomethiazole	249.9	17.177	Floctafenine	209.9
15.96	Perphenazine	255.8	17.192	Pimozide	205.2
15.977	Alimemazine metabolite 6	252.3	17.21	Virginiamycine	227.5
16.008	Nalidixic acid	258.2	17.235	Pristinamycine	227.5
16.035	Chlorpromazine	254.7	17.293	Propoxur	200.5
16.058	Carnitine	287.8	17.347	Trifluoperazine metabolite	1258.2
16.073	Midazolam metabolite	214.6	17.353	Triazolam	220.5
16.077	Mebendazole	209.9	17.357	Fluoxetine metabolite 1	200.5
16.142	Pentobarbital metabolite	200.5	17.357	Fluphenazine	259.4
16.143	4-Chlorobenzoic acid	200.5	17.358	Flupenthixol	228.7
16.167	Rifampicine	236.9	17.365	Embutramide	200.5
16.18	Carpipramine	200.5	17.417	Clonazepam	200.5
16.185	Fluoxetine	200.5	17.42	Secobarbital	200.5
16.19	Ethyl paraben	200.5	17.573	Chlortoluron	209.9
16.275	Naproxen metabolite 4	232.2	17.583	Rifabutine	208.7
16.288	Phenytoin	200.5	17.603	Glipizide	200.5
16.293	Cefuroxine peak 1	278.3	17.61	Clobazam metabolite 2	228.7
16.305	Dipropyline	207.5	17.653	Tiaprofenic acid	200.5
16.325	Zuclopenthixol	206.4	17.657	Chlorpropamide	200.5
16.368	Altazide	226.3	17.705	Mecoprop	200.5
16.377	Veratrole	201.7	17.735	Hydrocortisone	242.9
16.378	Benfluorex	200.5	17.747	Trifluoperazine	258.2
16.413	Chlorpromazine Metabolite	2254.7	17.777	Albendazole	218.1
16.433	Reserpine	218.1	17.8	Piretanide	200.5
16.437	Pentobarbital	200.5	17.833	Altretamine	229.9
16.442	Clomipramine	200.5	17.892	Flunindione	222.8
16.495	Estazolam	221.6	17.968	Carbaryl	220.5
16.512	Fluoxetine metabolite 4	200.5	18.062	Phenindione	226.3
16.52	Canrenoate	287.8	18.125	Naproxen metabolite 5	229.9
16.552	Cefuroxine peak 2	278.3	18.15	Parbendazole	207.5

(*Continued on p. 156*)

Table 1 (*continued*)

Retention time (min)	Spectrum name	γ_{max}	Retention time (min)	Spectrum name	γ_{max}
18.177	Atrazine	221.6	20.433	Propazine	221.6
18.202	β-estradiol	200.5	20.45	Monodesebuthylhalofantrine	258.2
18.262	Fenofibrate	200.5	20.5	Gliclazide	200.5
18.267	Clofibrate	200.5	20.51	Blood interference 14	342.6
18.268	Bezafibrate	200.5	20.652	Tamoxifen	200.5
18.275	Blood interference 5	200.5	20.68	Spironolactone	239.3
18.34	Propylparaben	200.5	20.768	Flavone	201.7
18.392	Griseofulvine	292.6	20.785	Blood interference 15	200.5
18.433	Blood interference 20	216.9	20.813	Diethyl ester of phthalic acid	201.7
18.44	Clorazepate	227.5	20.85	Rifamycine	225.2
18.48	Isosorbide	200.5	20.882	Diethylstilbestrol	200.5
18.503	Diuron	211.1	20.958	Amiodarone Metabolite	206.4
18.508	Tofisopam	204	20.968	Tritoqualine	214.6
18.558	Flunitrazepam	200.5	20.978	Chlorophacinone	200.5
18.562	Temazepam	200.5	21.027	Loflazepate	228.7
18.628	Cefpodoxime Peak 1	200.5	21.067	Clofibride	200.5
18.632	Bendroflumethiazide	208.7	21.135	Blood interference 4	240.5
18.66	Hematoporphyrine	347.4	21.16	Fenoprofen	200.5
18.695	Alminoprofene	201.7	21.18	Droperidol	202.8
18.725	Digitoxine	219.3	21.22	Ciprofibrate	200.5
18.823	Xipamide	218.1	21.253	Linuron	209.9
18.857	Xanthydrol	209.9	21.337	Pinaverium	213.4
18.887	Methylprednisolone	245.2	21.337	Flurbiprofen	200.5
18.945	Cefpodoxime Peak 2	200.5	21.503	Etodolac	225.2
19.055	Bumetanide	200.5	21.53	Miconazole	202.8
19.118	Terfenadine	200.5	21.565	Norgestrel	241.7
19.172	Biscoumacetate	208.7	21.638	Trifluperidol	200.5
19.19	Clobazam	229.9	21.652	Clotiazepam	211.1
19.202	Thiopental	285.5	21.748	Indometacine	201.7
19.258	Cinnarizine	200.5	21.852	Methylhydroxyprogesterone	242.9
19.263	Fenproporex	200.5	21.88	Toluene	215.8
19.292	Fenbufen	200.5	21.89	Prometrine	221.6
19.317	Flunarizine	200.5	21.915	Amiodarone	204
19.433	Chloropicrine	202.8	21.953	Glibenclamide	200.5
19.465	Benzene	209.9	21.968	Niflumic Acid	200.5
19.485	Nifedipine	236.9	22.015	Butylhydroxytoluene	200.5
19.503	Bepridil	202.8	22.05	Iodoform	200.5
19.548	Benzoyl-2 aminonitrothiazole	200.5	22.087	Nitrendipine	236.9
19.612	Blood interference 12	207.5	22.115	Diclofenac	200.5
19.628	Ketoprofen	200.5	22.153	Coumachlor	204
19.63	Diphacinone	200.5	22.248	Flutamide	200.5
19.79	Blood interference 3	200.5	22.352	Isradipine	200.5
19.815	3,4-Dichloroaniline	206.4	22.378	Tetrazepam	226.3
19.955	Meclozine	200.5	22.387	Chlorambucil	201.7
20.052	Acenocoumarol	204	22.495	Acetorphan	200.5
20.137	Econazole	200.5	22.525	Tioclomarol	200.5
20.15	Captodiamine	200.5	22.637	Minocycline	200.5
20.183	Penfluridol	200.5	22.752	Buclizide	200.5
20.327	Diazepam	200.5	22.943	Loratadine	200.5
20.332	Butylparaben	200.5	22.982	Lomustine	229.9
20.355	Coumafen	205.2	22.993	Halofantrine	258.2
20.358	Warfarine	205.2	23.082	Pentaerthrityle	200.5
20.373	Piperine	341.5	23.243	Blood interference 8	200.5
20.432	Clocinizine	200.5	23.342	Naphtalene	219.3

Table 1 (*continued*)

Retention time (min)	Spectrum name	γ_{max}	Retention time (min)	Spectrum name	γ_{max}
23.36	Prazepam	200.5	25.13	Disulfirame	216.9
23.412	Blood interference 6	219.3	25.363	Bromadiolone	202.8
23.652	Dinoseb	213.4	25.573	Trichlorocarbanilide	264.1
23.815	Ibuprofen	200.5	25.763	Diazinon	200.5
23.815	Megestrol	290.2	25.905	Almitrine	200.5
23.835	Progesterone	242.9	26.075	Benzbromarone	204
23.902	Anethole	205.2	26.24	Blood interference 9	233.4
23.92	Niclosamide	205.2	26.383	Cyclandelate	200.5
23.985	Xylene	218.1	26.477	Phorate	200.5
24.038	Norethisterone	240.5	26.54	Blood interference 10	235.8
24.098	Phenylbutazone	200.5	26.723	Fluoxetine metabolite 2	200.5
24.11	Chlormadinone	283.1	26.795	Di-syston	200.5
24.203	Medroxyprogesterone	241.7	27.235	Lacidipine	239.3
24.423	Felodipine	200.5	27.368	Methoxychlor	200.5
24.737	Bithionol sulfoxide	211.1	27.627	Piperonyle butoxide	202.8
24.86	Fusidic acid	200.5	27.658	Lufenuron	209.9
25.098	Blood interference 2	200.5			

3. Results and discussion

Due to its high separation power and its applicability to a large number of compounds, including non-volatile and thermolabile products, HPLC–PDA appears as a very valuable method for STA. Since the recent introduction of very reproducible column material, this method, originally criticized because of poor reproducibility of retention times (t_Rs), henceforward possesses a good selectivity due to stable t_R values.

3.1. Peak identification

Drug identification was carried out by using a retention time (t_R) pre-search technique which restricts the library search to a definite window around the t_R of the unknown peak and then by comparison of the unknown spectrum to spectra of all reference products comprised within the window. The time window currently in use is 6% around the nominal t_R. The software uses vector mathematics to compare spectra, and gives, with the peak identity, a match angle value. A match angle equal to 0.0° would mean a perfect match between the unknown and the library spectra. A peak identification result with a match angle below 4.0° can be considered as identification with great certainty, while a match angle higher than 8.0° is of poor certainty. Between these two values, results must be carefully examined depending on the general shape of the spectrum. For poor UV spectra like those of phenobarbital, lidocaine, phenytoine and zipeprol, match angles are generally superior to those of rich spectra containing several γ_{max} like antipsychotics, benzodiazepines, anticoagulants. In such cases t_R values are an important data.

One of the limitations of PDA detectors has been sensitivity, due to flow cells and optics design. Improved sensitivity enables the detection of drugs at low levels. Some authors estimate that below 20 mAU, obtaining decent spectra is almost impossible and that from 50 mAU detection is easy [13]. In fact, most of these authors did not evaluate their limits of identification. They expressed solely the limits of detection 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. Using that approach, Tracqui et al. [14] estimated their results in the range of 9 to 87 ng/ml for 27 neuroleptics, 2.5 to 15 ng/ml for 25 antidepressants and 8.5 to 54 for 26 benzodiazpines. Since the limits of detection and identification both depend on the optical quality of the apparatus and on the quality of the extraction method, we think it is preferable and more representative of the complete method to measure in some real cases the identification limit with the corresponding blood concentration. On one hand, the

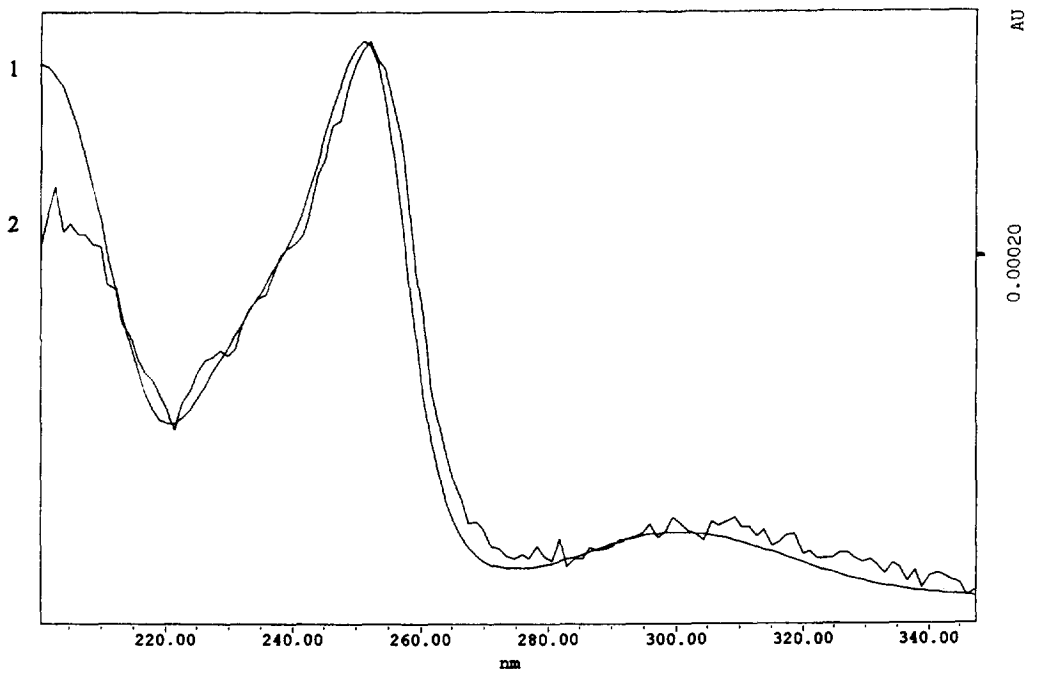

Fig. 1. Spectrum of an unknown compound at 0.29 mAU. 1 = promethazine, 2 = unknown peak.

richer a spectrum is, the better will be the library identification and, on the other hand, a more efficient extraction will give an higher absorbance value. Table 1 shows the results for some drugs obtained in real forensic cases. Fig. 1 shows the drug identification of prometazine at only 0.29 mAU. One can see that in some cases, the limit for library identification of drugs is equivalent to the limit of detection of the compound (Table 2). In practice, these limits were found sufficient to allow identification of most of the library's compounds at infra-therapeutic blood levels, and of course, a fortiori in poisoning cases.

Table 2
Limits of positive peak identification (PPI) obtained in real forensic cases (1 ml of whole blood extracted using Toxi-Tube A) and its comparison with the corresponding limits of detection

Compounds	PPI (mAU)	Blood concentration of PPI (ng/ml)	Limit of detection at γ_{max} (ng/ml)
Alprazolam[a]	0.90	8	5
Carbamazepine[a]	1.14	20	15
Ciprofloxacine[b]	0.62	7	5
Cyamemazine[a]	0.82	12	6
Furosemide[b]	0.60	16	7
Ibuprofen[a]	1.64	77	25
Lidocaine[b]	2.12	60	60
Metoclopramide[a]	0.70	7	7
Metoprolol[b]	2.07	62	30
Phenobarbital[a]	3.82	85	35
Zolpidem[a]	0.54	5	5

[a] Confirmed by GC–MS or GC–electron capture detection for alprazolam. [b] Confirmed by the clinical history.

3.2. Stability of t_R values and peak symmetry

Due to the lack of reproducible t_R values, some authors advocated the use of retention indices (I) rather than t_R values for the identification of unknown peaks. Bogusz and Wu [15–17] have proposed a computation of the I values of the substances against the corresponding 1-nitroalkanes scale. Introduction of I values has created the possibility of establishing inter-laboratory databases. However, with the recent appearance of more reproducible columns, low-delay volume pumps, and thermostated environments, the t_R values are clearly stable enough to ensure a substantial reduction of the time window parameter. Measured during 8 months of toxicological activity, the variability of t_R for some main compounds is listed in Table 3 ($n=16$, two measures per month).

Another important parameter is the obtention of symmetrical peaks. This is problematic for basic compound drugs of great interest, such as tricyclic antidepressants and tamoxifen which exhibit difficult chromatographic behaviours in reversed-phase separation. By using the Waters Symmetry column, peak-tailing effects were significantly reduced. It has two consequences, (a) improvement of drug separation and (b) reduction of the identification and detection limits (see Table 3 and Fig. 2).

The United State Pharmacopea (USP) tailing factor establishes the maximum permissible

Table 3
Variability of t_R for 20 main drugs expressed as the standard deviations (SDs) of nominal t_R values (%) during eight months ($n=16$).

Drugs	SD (%) of t_R
Acenocoumarol	0.9
Aciclovir	1.0
Alprazolam	1.3
Atrazine	1.5
Betaxolol	2.7
Carbamazepine	2.0
Cimetidine	4.2
Clomipramine	1.9
Cyclophosphamide	1.7
Diclofenac	1.3
Disopyramide	2.1
Floctafenine	1.4
Hydrochlorothiazide	4.3
Pefloxacine	1.4
Phenobarbital	1.8
Quinine	3.6
Thioridazine	2.3
Tiapride	4.2
Toloxatone	2.5
Zopiclone	1.7

Table 4
USP tailing factor of some basic drugs

Name of the compounds	USP tailing factor (T)
Sulpiride	0.956
Tiapride	1.423
Moclobemide	1.069
Viloxazine	1.043
Trazodone	1.049
Medifoxamine	1.545
Doxepine	1.071
Dosulepine	1.102
Maprotiline	1.038
Clomipramine	1.062
Tamoxifen	1.082

Table 5
Main pharmacological classes of drugs and metabolites listed in the library

Drugs classes	Number of drugs present
Alkaloids	34
Antiarythmics	8
Antibiotics	54
Anticoagulants and rodenticides	12
Antidiabetics	9
Antiepileptics	8
Antihistaminics	17
Antimalarials	11
Antiparasitics	20
Anorexigens and stimulants	9
Barbiturates	10
Benzodiazepines and other anxiolytics	29
β-Blockers and β-agonists	18
Central analgesics and antagonists	14
Cytostatics	9
Diuretics	11
Drugs of abuse	16
Interferences	20
Miscellaneous	177
Neuroleptics	55
NSAIDs	23
Other drugs with cadiovascular activity	29
Pesticides	27
Steroids	16
TCADs and MAOIs	48

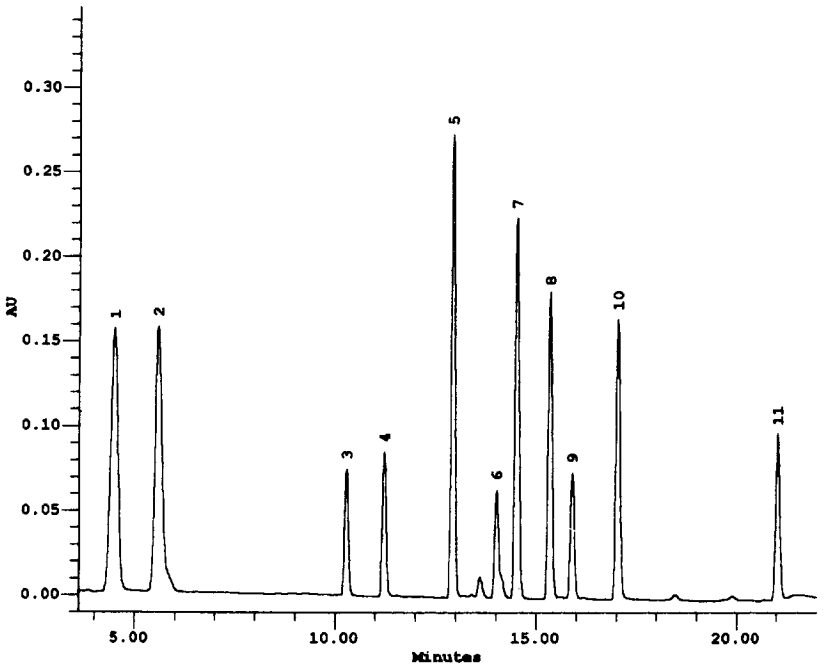

Fig. 2. Chromatogram monitored at 220 nm of a pure mixture of some basic drugs (0.5 μg each injected). Separation column: Waters Symmetry C_8, 250 mm length × 4.6 mm I.D. (5-μm particle size) equipped with a 20-mm guard column (Waters Symmetry C_{18}). Separation conditions are as follows: column temperature 30°C, solvent A = phosphate buffer (pH = 3.8), solvent B = acetonitrile; step gradient is 15% B for 6.5 min, then 35% until 25 min, then 80% B for 3 min. The flow-rate of the mobile phase was 1 ml/min for 6.5 min, then linear increase to 1.5 ml/min from 6.5 min to 25 min and hold for 3 min. Peaks: 1 = sulpiride, 2 = tiapride, 3 = moclobemide, 4 = viloxazine, 5 = trazodone, 6 = medifoxamine, 7 = doxepine, 8 = dosulepine, 9 = maprotiline, 10 = clomipramine and 11 = tamoxifen.

asymmetry of the peak. For a symmetrical peak, the tailing factor, T, is unity and the value of T increases as tailing becomes more pronounced. T is defined by the mathematical expression:

$$T = \frac{W}{2F}$$

where T = tailing factor, W = peak with at 5% of peak height, F = width of line from peak start to t_R at 5% of peak height. Results are given in Table 4 (see also Fig. 2). Symmetry can be considered excellent for all compounds except for tiapride and medifoxamine.

3.3. Drugs listed in the library

Table 5 summarizes the different drugs listed in the library by pharmacological classes. To avoid or minimize the risk of misinterpretation with normal blood components, the library also incorporates 20 interfering substances. These substances were isolated from blood and urine extracts obtained from drug-free volunteers. Specificity of the library was judged excellent since only three pairs of drugs are really exposed to the risk of confusion. Such problems could arise with acenocoumarol and warfarine, which elute within a very narrow time window and exhibit very similar UV spectra, and also for fenofibrate–clofibrate and cyamemazine–thioproperazine.

For two drugs (dextropropoxyphene and dextromoramide) we have observed a slight modification of the UV spectra attributed to a matrix effect. Because these compounds exhibit poor UV spectra, they are not correctly identified by the library. For this reason, the spectra obtained in real cases were stored in the library together with the pure standard and their main metabolites.

When an analytical report suggests the presence of drugs of abuse, their presence in the sample must systematically be confirmed by GC–MS. However, and independently to non-volatile compounds, we

personally think, that the identification of certain pharmaceuticals like tricyclic antidepressants or neuroleptics, is of better quality by the present method, than by GC–MS analysis (main ions m/z = 44, 58 or 72 are of poor selectivity in a blood extract as compared to rich spectrum with several γ_{max} for the same drug). Moreover, especially in blood samples, due to lower endogenous interference, HPLC–PDA technology allows an easier identification of those pharmaceuticals than GC–MS.

4. Applications

4.1. Case report 1

A 40 year-old man known by the police as a drug addict was found dead in his apartment. Some boxes of pharmaceuticals (moclobemide, aspirin, paracetamol, dextropropoxyphene, nordazepam, phenobarbital) were discovered in the living room together with empty bottles of alcohol. Blood and urine were taken at the autopsy for toxicological analysis. Fig. 3 displays the chromatogram obtained from an extract of 1 ml of urine from the decedent (using a Toxi-Tube A). HPLC–PDA screening in urine gives the following results: morphine = 895 ng/ml, moclobemide = 19.6 μg/ml, minaprine = 0.7 μg/ml, phenobarbital = 2.6 μg/ml, oxazepam = 9.1 μg/ml and nordazepam = 1.9 μg/ml. Salicylic acid was also identified but quantified by an appropriate colorimetric method (150 μg/ml). Additionally detected in the urine were: codeine = 77 ng/ml and THCCOOH = 11 ng/ml by GC–MS and ethanol = 1.25 mg/ml by headspace GC–flame ionization detection.

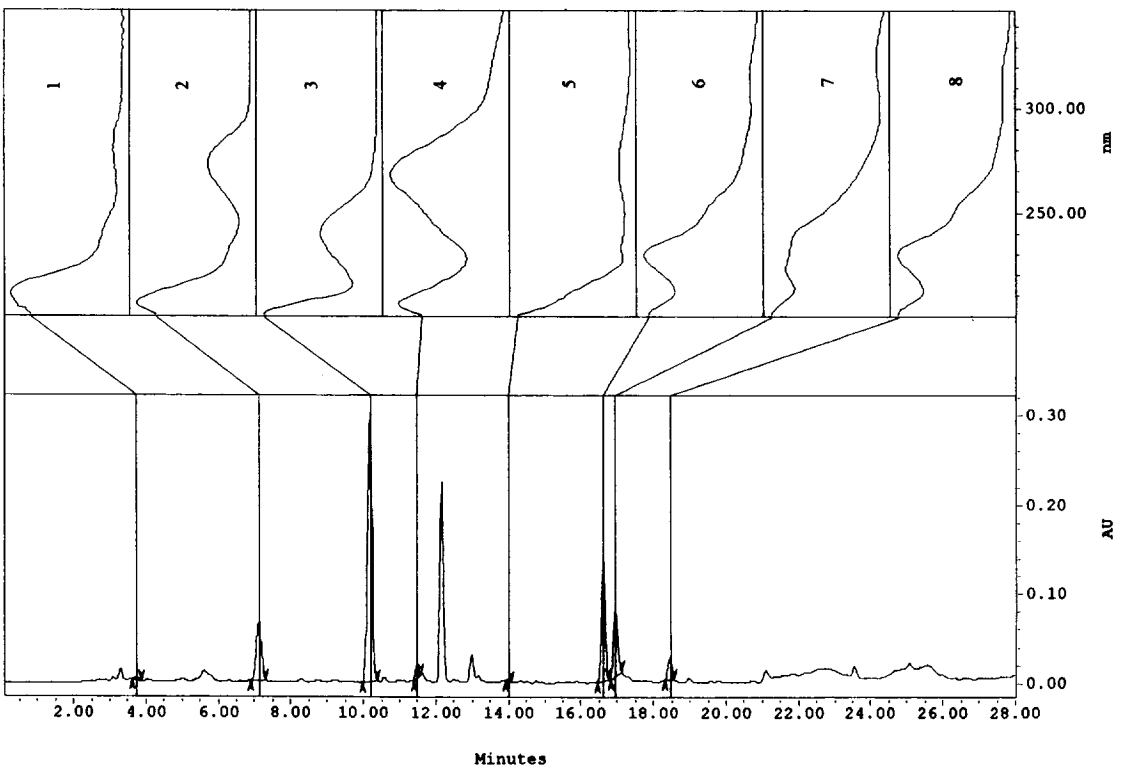

Fig. 3. Chromatogram monitored at 210 nm of an extract of 1 ml of urine of the deceased man of forensic case 1. Peaks: 1 = morphine, 2 = caffeine, 3 = moclobemide, 4 = minaprine, 5 = phenobarbital, 6 = oxazepam, 7 = benzophenone and 8 = nordazepam. For conditions, see Fig. 2.

4.2. Case report 2

A 32 year-old man was found dead at his home, with neither signs of violence or needle marks on the corpse. He was treated for psychiatric disorders with amitriptyline, amisulpride and diazepam and received also fluconazole and betaxolol. Although the coroner did not notice visceral congestion when he performed the post-mortem examination of the corpse, blood and urine were taken for toxicological analysis. 1 ml of whole blood extracted using a Toxi-Tube A revealed the presence of: amisulpride = 0.54 μg/ml, metoclopramide = 0.11 μg/ml, fluconazole = 0.84 μg/ml, betaxolol = 0.01 μg/ml, nortriptyline = 0.19 μg/ml, amitriptyline = 0.45 μg/ml, nordazepam = 0.26 μg/ml and diazepam = 0.08 μg/ml (see Fig. 4). No other pharmaceuticals, toxicants, pesticides, drugs of abuse, solvents, cyanide, carbon monoxide were detected. However, glucose was measured in the blood sample at 17.9 g/l (a normal result is contained between 0.70 and 1.05 g/l) and in urine at 41.4 g/l, which was the official cause of death. This interesting example shows that a large variety of drugs can be easily identified and quantified by the described method even when present at low therapeutic concentrations.

5. Conclusion

Although originally developed for qualitative approach, HPLC–PDA detection can be easily adapted for sensitive quantitative measurements when extracting chromatograms at specific detection wavelengths from the three dimensional acquisition, as with a 'normal' UV detector. Newly encountered compounds can easily be added to the library as well as their metabolites, with matrix effects and interfer-

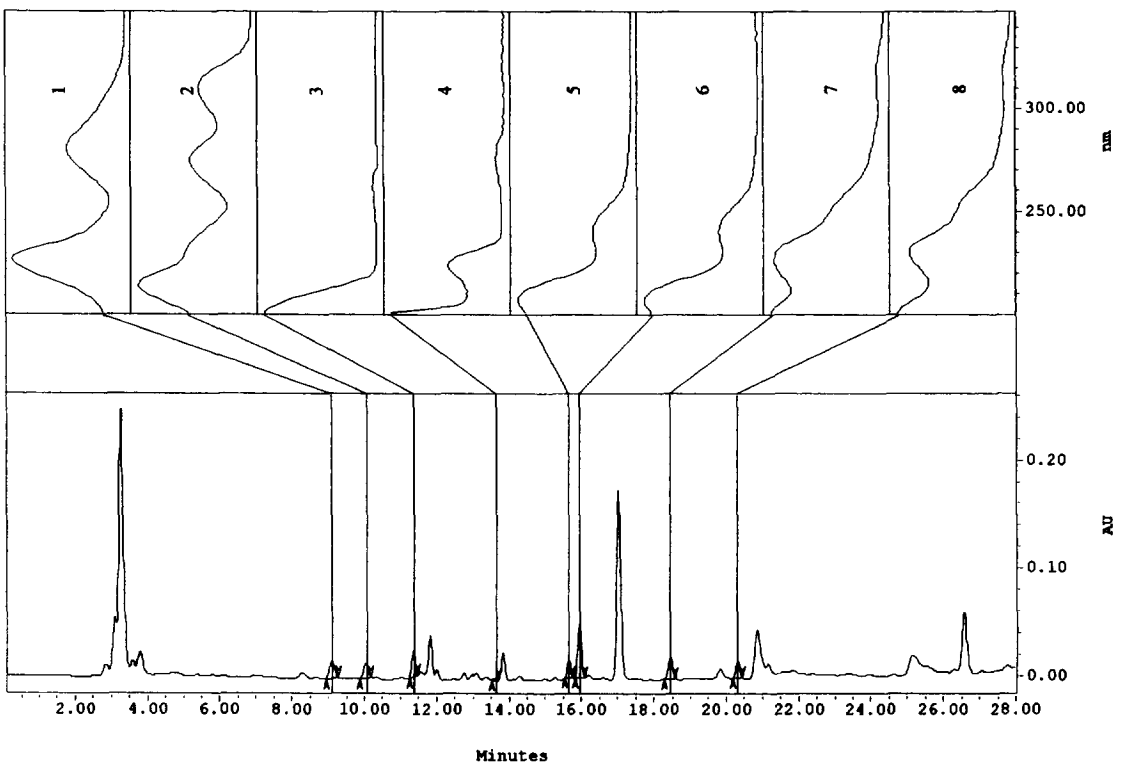

Fig. 4. Chromatogram monitored at 210 nm of an extract of 1 ml of whole blood of the deceased man of forensic case 2. Peaks: 1 = amisulpride, 2 = metoclopramide, 3 = fluconazole, 4 = betaxolol, 5 = nortriptyline, 6 = amitriptyline, 7 = nordazepam and 8 = diazepam. For conditions, see Fig. 2.

ence. The described method allows a powerful screening of a wide variety of drugs and toxicants. Although immunoassays remain irreplaceable tools for initial screening, the combination in a second step, of GC–MS and HPLC–PDA represent a classic and necessary work-load for every toxicological laboratory.

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