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TOXICOLOGICAL SCREENING METHOD BY HPLC-DAD WITH LOCAL SPECTRAL LIBRARY AND "HOME-MADE" INTERNAL STANDARD

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ABSTRACT

A liquid-liquid extraction technique and HPLC-DAD method using a C18 column, 5µm (150x4.6mm i.d.) were developed for a toxicological drug screening. A home-made "N-acetylparoxetine" is used as internal standard. A local UV spectra library of over 130 drugs and some of their metabolites was created. This library covers the main therapeutic classes involved in intoxication cases. The developed procedure is successfully applied to different biological samples (serum, urine, gastric fluid and post mortem whole blood) and allows rapid and simultaneous detection and identification of a large number of substances and their semi-quantification in serum.

Keywords: HPLC-DAD, LLE, drug poisoning, systematic toxicological analysis.

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1. INTRODUCTION

In toxicological analysis, the searched compounds are often unknown and clues to guide the analysis are insufficient or absent. It also happens in some poisoning cases that the clinical progress doesn't relate to the suspected toxics or the previously identified ones [1]. In such cases, the toxicologist must achieve a systematic toxicological analysis (STA) by performing

a number of analysis to detect, identify and quantify the toxics or in contrary to exclude their presence [2].

The sample preparation which aims to make the analyte compatible with analytical system is a key step in STA and has a major impact on the quality of the analysis [3,4]. Many efforts have been made to optimize this step where the techniques can be classified into pre-treatment techniques, namely the hydrolysis of conjugates and protein precipitation, extraction techniques in liquid phase (ELL) and solid phase and derivatization. The choice of work-up procedures depends on the nature and quantity of the sample, the suspected toxic and the analytical method used for screening [3-14].

For this aim, a variety of analytical methods are available, but none can detect all toxics rapidly and at low cost [1]. A laboratory must set an analytical procedure involving several complementary methods for the detection and quantification of the widest possible panel of molecules and their metabolites with high specificity and sensitivity. The selected procedure depends on the cases to be solved. It is dictated by the expectations and priorities set by the clinician and should be adapted to the available equipment but also to the nature and quantity of samples [7,15].

Colorimetric, spectrophotometric and enzymatic methods have the advantage of being accessible, rapid and easy to perform but are limited to certain toxics and lack sensitivity and specificity [15-18]. Immunoassays cover a greater number of legal and illegal drugs with higher sensitivity and specificity and are both easy and appropriate to emergency. However, in addition to their relatively high cost, the results should be carefully interpreted because of many pitfalls that must be known and taken into account especially when the results are not confirmed by valid confirmation procedures [19,20].

Nowadays, chromatographic methods coupled with various detection systems hold the most important place in STA. They allow to characterize simultaneously a wide range of molecules with a higher specificity and sensitivity compared to previous methods [3,21]. The GCMS remains the reference method or "Golden Standard" for STA [7,22]. With the advent of promising high resolution spectrometric techniques, LCMS is gaining more interest as the majority of new drugs are more polar and less volatile which makes it a good alternative to the

GCMS limitations [7,22-26]. However, many laboratories in the world cannot afford the high cost of CGMS and LCMS equipment [27] and use some more accessible methods such as HPTLC [9,16,28,29] and HPLC-DAD. This last one is considered nowadays as a very effective method for its high robustness, easy handling, lower costs, exact spectral reproducibility, long-term reproducibility of concentration-absorbance relationship and low sensitivity to matrix interferences [29]. Therefore, it covers simultaneously and with a high specificity a large number of molecules with very different physicochemical properties and allows a semi-quantitative determination of concentrations [8,25,30,31].

This article describes the optimization of a liquid-liquid extraction procedure and toxicological screening method using HPLC-DAD with the creation of a local library over 130 of the most implicated drugs in poisoning and certain of their metabolites. Another peculiarity of our method is the use of N-acetylparoxetine as a home-made internal standard.

1. MATERIALS AND METHODS

2.1 Materials

2.1.1. Reagents

Methanol (Sigma Aldrich), dichloromethane (Panreac), pure heptane (Cheminova), isopropanol (Panreac), ammonium chloride pure (Buchmann), phosphoric acid (Prolabo), pure quality Acetonitrile HPLC gradient (Sigma Aldrich), potassium dihydrogenophsphate (Panreac), ammonia (Cheminova), pyridine (Merck), acetic anhydride (Prolabo).

2.1.2. Drug standards

Powders of active substances (purity ≥ 98%) including paroxetine used to prepare the internal standard were obtained from various pharmaceutical laboratories mainly Saidal and Biopharm. Tablets and capsules were provided by Central Hospital of Army's pharmacy. Lyophilized serums of Chromsystems® standards of 26 molecules (6 tricyclic antidepressants, 7 benzodiazepines, 6 antiepileptics and some of their metabolites) were also used to establish the local library.

2.1.3. Instrumentation

The analysis is performed using a HPLC system with pumping system (Spectra System P1000XR), column oven (EPPENDORF CH-500), HPLC column C18 5µm (150x4.6mm) (THERMO) protected by guard column C18,5µm (10x4 mm i.d.) (HYPERSIL GOLD) and a diode array detector (Spectra System UV6000LP). The Data acquisition is handled by ChromQuest^{TM} software v4.2.34 (THERMO).

2.2. METHOD

2.2.1. Synthesis of the internal standard

The N-acetylparoxetine is not a metabolite of paroxetine. It is therefore chosen as internal standard and synthesized in our laboratory by complete acetylation of 10 mg of pure paroxetine in 1 ml of pyridine /acetic anhydride (1:1 v/v). After 30 minutes at 70°C, the mixture is evaporated under nitrogen stream and the residue is dissolved in 10 ml of methanol. The working solution is a dilution of the stock solution to the fifth in water/methanol (50:50 v/v). Both solutions are stored at +4°C and are stable over 3 months.

2.2.2. Preparation of standard solutions

Stock solutions of 1g/L of active substance are prepared in methanol from powders and pharmaceutical forms. Uncoated tablets and the contents of capsules are weighed then ground to uniform powder [23]. These stock solutions are used to prepare mixtures in water/methanol (50:50 v/v) at a concentration of 10 mg/L for each molecule.

The Chromsystems standards are reconstituted prior to the analysis and undergo extraction according to the optimized procedure. All the solutions are stored at +4°C.

2.2.3. Chromatographic conditions

The mobile phase is a mixture of Acetonitrile and 25mM phosphate buffer KH_2PO_4 pH 2.6. The elution is performed in gradient mode to a constant flow rate of 1.3 ml/min (15% Acetonitrile for 2 min and increases linearly to 75% in 34 min). The column temperature is set at 28°C. The acquisition wavelength is 210 nm and spectral scanning is carried out in the range 200-400 nm with a resolution of 1 nm.

2.2.4. Sample preparation

To one ml of serum, urine or gastric content is added successively 1 ml of NH_4Cl buffer (pH = 9.5), 200µl of N-acetylparoxetine and 8 ml of dichloromethane/heptane/isopropanol (60:26:14 v/v/v). The extraction is carried out for 10 min. After centrifugation, the organic phase is evaporated to dryness under nitrogen stream at 40°C. The residue is dissolved in 200 µl of water/methanol (50:50 v/v) and centrifuged. 50 µl of supernatant are injected.

For postmortem blood, a protein precipitation by Acetonitrile is achieved prior extraction.

2.2.5. Establishing local library

Knowing the impact of environmental and experimental conditions on UV-spectra and retention time, it is recommended to use in-house libraries for STA by HPLC-DAD [8,31]. To set our local library, mixtures of about 10 molecules in methanol were analyzed. The UV spectrum, retention time and the name of each identified molecule are stored in a database used for the identification of the molecules found in biological samples or compounds found beside the patient (powder, tablets, syringe contents).

2.2.6. Determining the liquid-liquid extraction yield

5 mixtures of 14 molecules were prepared in blank serum. The extraction yield was determined for each molecule from the peak areas at 210 nm as following:

$$\text{Yield \%} = \frac{\text{peak area of blank serum spiked then extracted}}{\text{area obtained for the blank serum extracted then spiked}} \text{ x100}$$

2. RESULTS AND DISCUSSION

3.1. Synthesis of the internal standard

The following Figures 1 and 2 show the chromatograms of the injections of paroxetine solution before and after acetylation.

In STA, since the majority of searched substances are drugs; it is preferable that the internal standard is neither a therapeutic molecule nor its metabolite. Therefore, the home-made N-acetylparoxetine is used as internal standard since the acetylation is not a metabolic pathway of paroxetine and shifts the retention time without modifying the UV spectrum. The N-acetylparoxetine is prepared in our laboratory by a total acetylation of pure paroxetine

through a very simple procedure. The total acetylation of paroxetine and the stability of the solutions are monitored by HPLC-DAD and GC-MS.

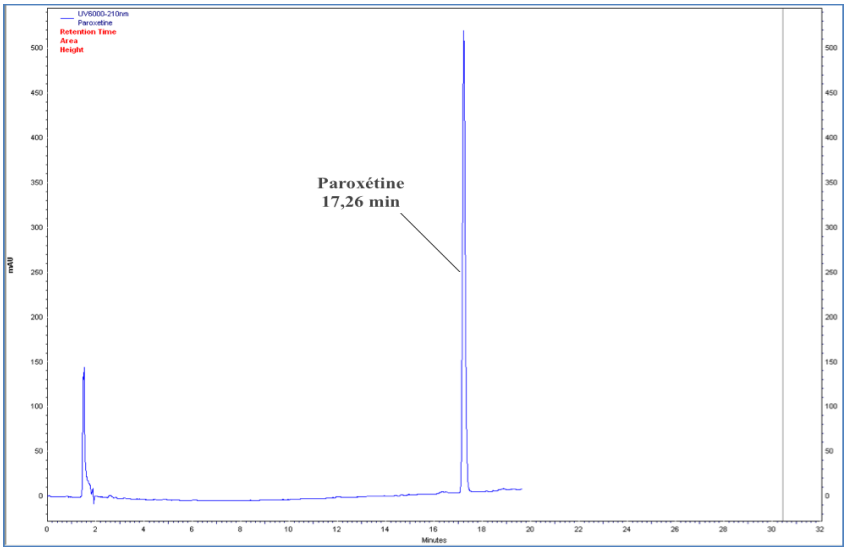

Fig.1. Chromatogram of a pure paroxetine solution before acetylation

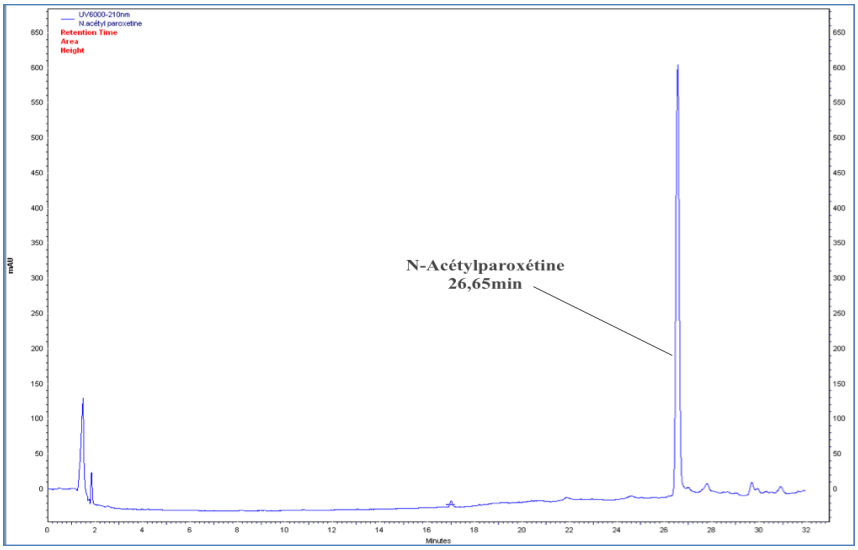

Fig.2. Chromatogram of a pure paroxetine solution after acetylation

3.2. Establishing local library

In Fig. 3 to 7, we present the chromatograms of certain mixtures of molecules. Our method allows within 35 minutes of analysis, the simultaneous detection and identification of a large number of molecules with a high resolution and reproducibility. An in-house library of over 130 molecules of toxicological concerns was established from powders of pure active substances, pure standards and pharmaceutical forms. UV spectra obtained at the top of the peaks are similar to reference spectra with a shift of +/- 2nm of the absorption maxima for

certain molecules [32]. Our library also covers some metabolites whose identification simplifies the interpretation of chromatograms and provides useful information in some poisoning cases.

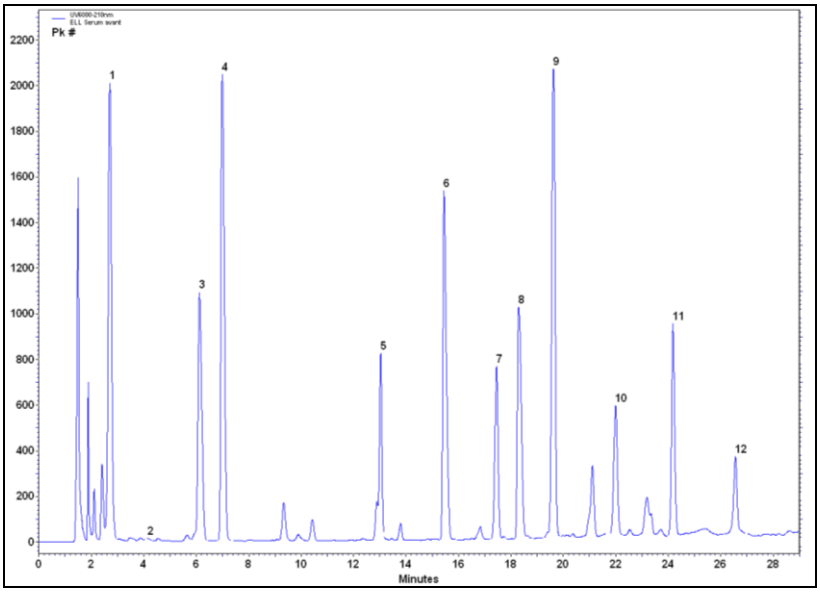

Fig.3. Chromatogram of the mixture 1. Identification of peaks: 1, Olanzapine; 2, Oxytetracycline; 3, Acebutolol; 4, Alfuzocin; 5, Prednisone; 6, Haloperidol; 7, Metronidazole; 8, Dextropropoxyphene; 9, Griseofulvine; 10, Gliclazide; 11, Rifampicin; 12, Ibuprofen.

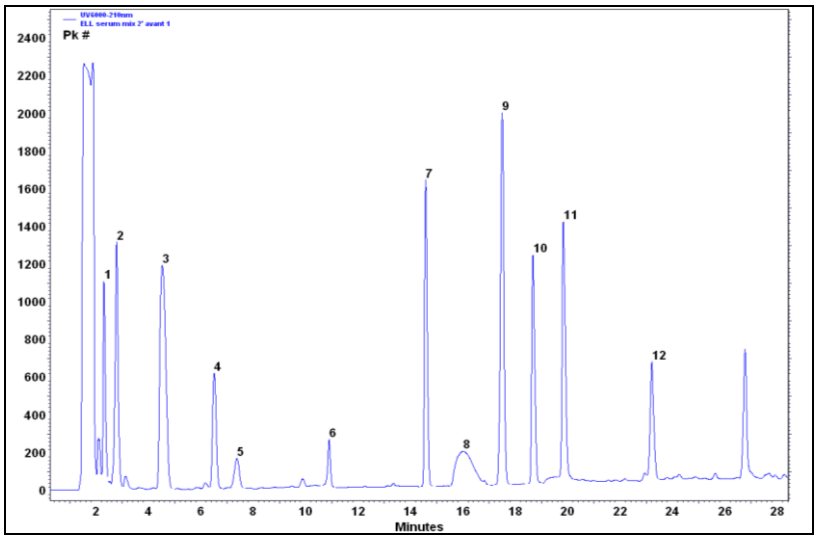

Fig.4. Chromatogram of the mixture 2. Identification of peaks: 1, Paracetamol; 2, Doxilamine; 3, Ropiniril; 4, Fluconazole; 5, Dapsone; 6, Doxycycline; 7, Dihydro-ergotamine; 8, Ramipril; 9, Clorazepate-Na; 10, Fexofenadine; 11, Chlorpromazine; 12, Valsartan

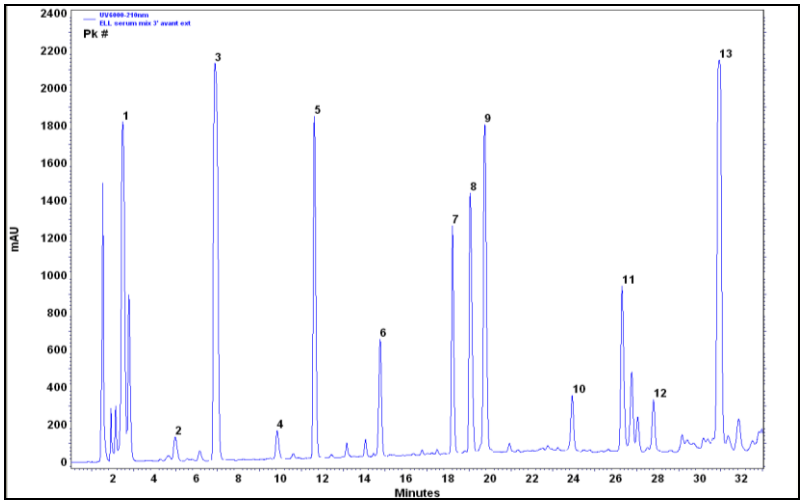

Fig.5. Chromatogram of the mixture 3. Identification of peaks: 1, Codeine; 2, Cocaine; 3, Lamotrigine; 4, Salicylic acid; 5, Ketotifen; 6, Piroxicam; 7, Amlodipine; 8, Trimipraminel; 9, Loratadine; 10, Spironolactone; 11, Miconazole; 12, Glimeperide; 13, Fussidate-Na

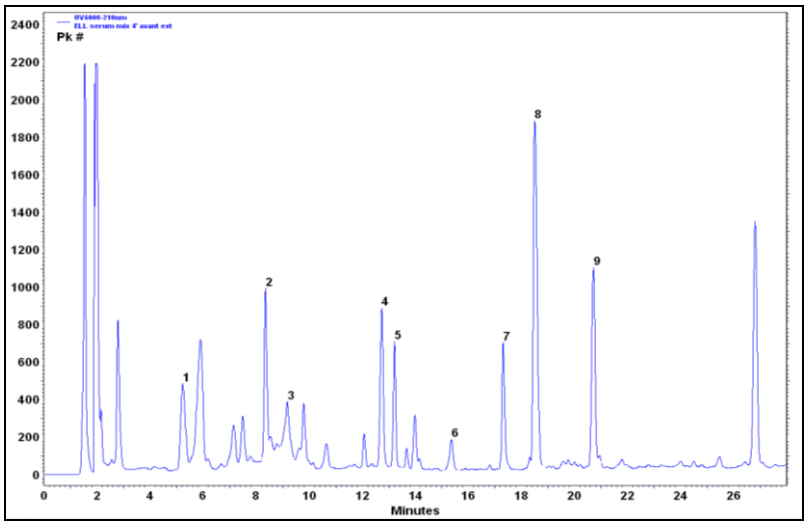

Fig.6. Chromatogram of the mixture 4. Identification of peaks: 1, Atropine; 2, Omeprazole; 3, Risperidone; 4, Lansoprazole; 5, Hydrocortisone; 6, Furosemide; 7, Paroxetine; 8, Amitriptylline; 9, Ketoprofen

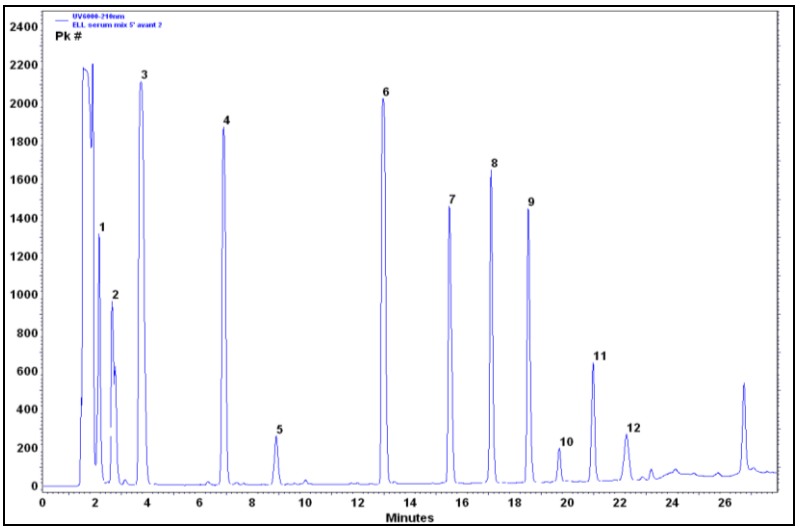

Fig.7. Chromatogram of the mixture 5. Identification of peaks: 1, Sulpiride; 2, Pseudoephedrine; 3, Trimethoprim; 4, Pentoxfylline; 5, Sulfamethoxazole; 6, Propranolol; 7, Flecainide; 8, Ketoconazole; 9, Levomepromazine; 10, Mycophenolic acid; 11, Naproxene; 12, Clopidogrel

3.3. The extraction yields

The simple and rapid optimized liquid-liquid extraction procedure allows simultaneous extraction of several molecules with different physicochemical properties from 1ml of sample or less, while reducing interferences of the matrix. The extraction yields calculated for the main molecules involved in acute poisoning are stable and suitable for the STA.

Paracetamol and NSAIDs are the main molecules with low extraction efficiency because these acid molecules are weakly extracted at the basic pH of our procedure. This low recovery is not considered as a drawback in case of poisoning because these substances have a large therapeutic dose leading to high concentrations in biological samples. On the contrary, it is perceived by some authors as an advantage because it avoids the saturation of the detector that has a negative impact on the identification [8,33].

During 3 successive days, the extraction yield was calculated for 58 molecules among the most involved drugs in acute poisoning (Table 1). The average yield is less than 50% for 14 molecules and over 60% for the remaining 44 molecules of which 31 have a yield greater than 70%. The Coefficient of variation (CV) is less than 30% for all molecules except for 8, which shows a good stability of the extraction and ensures the semi-quantification of the molecules.

Table 1. Extraction yields, coefficient of variation and detection limits (DL)

Molecules	Average yield (%)	CV (%)	DL (mg/l)	Molecules	Average yield (%)	CV (%)	DL (mg/l)
Acebutolol	94,72	7,1	0,04	Ketoprofen	37,56	22,37	0,04
Salicylique acid	7,4	14,62	0,36	Lamotrigine	87,5	23,91	0,02
Alfuzocin	89,52	3,05	0,02	Lansoprazole	146,62	3,33	0,05
Amitriptyline	68,94	10,44	0,01	Levomepromazine	64,51	0,36	0,03
Amlodipine	76,08	38,27	0,04	Loratadine	67,71	15,88	0,04
Atropine	75,21	7,28	0,12	Metronidazole	62,97	3,93	0,07
Carvedilol	92,07	13,55	0,02	Miconazole	39,19	22,1	0,13
Chlorpromazine	54,46	19,62	0,04	Mycophenolic Acid	6,25	0,45	0,26
Clopidogrel	53,37	44,34	0,19	Naproxene	23,18	5,34	0,07
Clorazepate-Na	94,96	5,73	0,02	Olanzapine	94,09	17,45	0,02
Cocaine	26,96	33,96	0,59	Omeprazole	136,9	0,11	0,04
Codeine	102,89	34,68	0,03	Paracetamol	37,15	25,34	0,05
Dapsone	77,51	4,58	0,22	Paroxetine	94,48	2,19	0,06
Dextropropoxyphene	68,09	1,27	0,05	Pentoxifillyne	106,91	13,26	0,02
Dihydro-ergotamine	89,89	2,7	0,03	Piroxicam	28,6	5,79	0,09
Doxilamine	100,14	2,4	0,04	Prednisone	95,59	3,19	0,06
Doxycycline	26,98	2,46	0,20	Propranolol	86,33	5,31	0,02
Fexofenadine	73,24	15,21	0,04	Pseudoephedrine	76,41	1,63	0,05
Flecainide	93,98	9,41	0,03	Ramipril	55,38	35,12	0,26
Fluconazole	91,86	8,3	0,09	Respiridone	49,72	45,89	0,22
Furosemide	7,8	13,18	0,24	Rifampicin	74,24	7,23	0,05
Fussidate-Na	94,87	18,38	0,14	Ropinirol	98,45	10,15	0,04
Gliclazide	71,44	0,44	0,07	Simvastatin	50,87	17,63	0,26
Glimeperide	63,07	19,68	0,07	Spironolactone	6,46	22,29	0,11
Griseofulvin	90,09	8,77	0,02	Sulfamethoxazol	7,49	0,54	0,18
Haloperidol	80,53	9,42	0,03	Sulpiride	59,93	38,23	0,04
Hydrocortisone	67,68	8,24	0,08	Trimethoprime	110,09	26,69	0,02
Ibuprofen	54,53	0,14	0,07	Trimipramine	66,78	3,53	0,04
Ketoconazole	83,63	5,58	0,03	Valsartan	22,59	19,4	0,10

The extraction yield cannot be calculated for Chromsystems standards molecules. However, they are all extracted and detected at therapeutic concentrations (Table 2).

Table 2. Limits of detection of Chromsystems standards molecules

Molecules	DL (mg/l)	Molecules	DL (mg/l)
Amitriptyline	0,01	Imipramine	0,02
Bromazepam	0,03	Nitrazepam	0,01
Carbamazepine	0,04	Norclozapine	0,01
Chlordiazepoxide	0,01	Nordiazepam	0,01
Clobazepam	0,01	Nordoxépine	0,01
Clozapine	0,01	Nortriptyline	0,02
Desipramine	0,02	Oxazepam	0,01
Diazepam	0,01	Phenobarbital	0,11
Doxepin	0,01	Phenytoin	0,06
Carbamazepine Epoxyde	0,02	Primidone	0,08
		Ethosuximide	0,68

3.4. The limits of detection (LD)

The detection limits are calculated at a signal-to-noise ratio of 3 at 210 nm after extraction [5] (Tables 1 and 2). In STA, the LD should be lower than the toxic concentration. The LDs of all tested drugs meet this requirement and most of them are detected at therapeutic concentrations, except for atropine that has a very low toxic concentration.

The new generation DADs are able to detect the substance in 98% of cases at sub-therapeutic levels hence at infra-toxic concentration [16]. This is confirmed for all the tested molecules for which the LDs are below toxic concentrations except for atropine. Moreover, the majority are detected at therapeutic concentrations allowing the identification of the patients' treatment and a drug monitoring as a first approach in therapeutic failure.

3.5. The semi-quantification

In STA, the quantification cannot be carried out for a large number of substances. However, a semi-quantitative approach is possible due to the stability in time of all optical measurements and the general proportionality between the concentration and the UV absorption [30].

The average peak area obtained for each molecule after extraction were saved in a database for further semi-quantification using the following formula:

$$C_{sample} = \frac{\text{peak area in patient sample}}{\text{average area of the standard}} \times C_{standard}$$

The systematic error due to the removal of the tablet's coat and weighing is acceptable in poisoning cases since a certain deviation from the real value can be tolerated [30].

3.6. Identification of metabolites

In addition to the parent compounds, many metabolites are frequently found, especially in the urine. Few of them are included in commercial libraries or available as pure standards [23]. HPLC-DAD offers the possibility to identify certain metabolites of a substance present in the same sample through the similarity of their UV spectrum and the characteristic shift of the retention time [8.30]. Thus, beside the Chromsystems metabolites, some urinary metabolic profiles obtained from previous intoxication cases are registered in our library [8].

3.7. Power of identification of molecules

The specificity of this method is valued for its ability to identify and differentiate molecules belonging to the same chemical family that have very similar UV spectrum (exp. Valsartan, Irbesartan and Losartan) and also certain molecules which have no characteristic UV spectrum.

In Fig. 8, a chromatogram of a patient serum with a peak showing no characteristic UV spectrum. Checking peak purity (systematically realized for each peak) reveals a co-elution of carbamazepine-epoxide (active metabolite of carbamazepine) with phenobarbital in a single peak. Phenobarbital is identified with a similarity index SI = 0.9998 in the front of the peak and carbamazepine-epoxide with a SI = 0.9968 in the end of peak. Thus, this method allowed the detection and identification of the patient's treatment namely omeprazole, phenobarbital and carbamazepine at therapeutic concentrations.

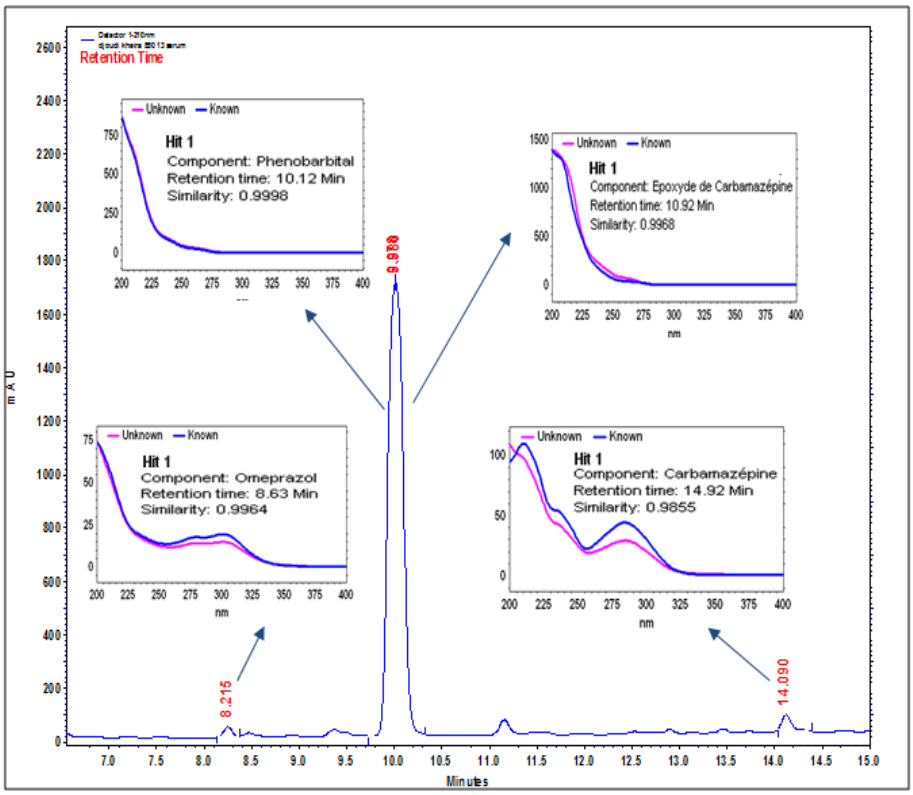

Fig.8. Chromatogram of a patient's serum

This method provides also a fast and effective estimation of serum concentration which is very advantageous in emergency cases. The reliability of the semi-quantitative approach is verified by the dosage performed on Chromsystems quality control where concentrations calculated were all included in the reference range suggested by the manufacturer. For the other molecules, the results are compared to those obtained by immunoassay which gives very close values.

4. CONCLUSION

We optimized a method of screening by HPLC-DAD in order to improve our analytical strategy and make it more efficient and adapted to emergencies. We established a local library of over 130 drug molecules and some of their metabolites and use an in-house N-acetylparoxetine, prepared by a simple procedure, as internal standard We have also optimized a liquid-liquid extraction procedure that allows a simultaneous extraction of several molecules with stable and decent recoveries and a detection limit that cover the toxic concentrations.

Our optimized method has a high specificity and stability of retention times. It is applied successfully on various biological samples and other products found close to the patient. It is very advantageous in clinical and post-mortem analysis when the history is unknown or when the sample volume is reduced. It has a decisive role for the molecules not covered by other methods (e.g., colorimetric tests, immunoassay) and the confirmation of positive or doubtful results obtained by these methods. It is also very useful for solving cases of ambiguous poly-drug poisoning and some treatment adherence cases.

Finally, many other molecules and substances of toxicological interest that can be analyzed by HPLC-DAD are not included in our library. Therefore, our database is regularly updated and expanded to include new substances upon doctors' request and when new standards are available.

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