

Drugs Identification in Urine, Bile and Gastric Contents using Thin Layer Chromatography in Multiple Screening Systems

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Abstract

A method of simultaneous identification of 25 molecules in human urine, bile and gastric contents using liquid-liquid extraction followed by thin layer chromatography (TLC) using multiple screening systems is described. The analytes were extracted at 25°C under isocratic conditions using chloroform after acidification with 1 to 2 drops of HCl 6 N for 10 mL of the biological sample, and dichloromethane after alkalization with 1 to 2 drops of NaOH 10 N for 10 mL of the biological sample. Employing LLE, the best conditions were achieved with double extraction of 10 mL of the biological sample, pH=9.5 for alkaline extraction and pH=2 for acid extraction. The organic extractums were filtered and dehydrated using anhydrous sodium thiosulfate powder and concentrated after evaporation of the organic solvents at 65°C. The extraction residues were solubilized in 500 µl of methanol and spotted with the molecules of reference onto four TLC plates (10 cm × 10 cm). The TLC plates were put into twin-through development chambers previously incubated 30 minutes for saturation namely TA (methanol:ammoniac 5% (50:0.750, v/v)), TD (chloroform:acetone (40:10, v/v)), TE (Ethyl acetate:methanol:ammoniac (42.3:5:2.5, v/v/v)), TB (cyclohexan:toluene:diethylamine (37.5:7.5:5, v/v/v)). The mobile phase migrates by capillarity through the stationary phase, driving at different speeds the molecules to be separated. The migration time (several minutes) depends on various parameters. When the solvent front has moved through a distance considered as sufficient (a few centimetres), the TLC plates were removed and dried, then exposed to ultraviolet light, the retardation factors R_f of each visible spot was measured. Some chemical processes might also be used to reveal spots. The total number of substances present in the biological sample was determined by counting the number of spots found on each TLC plate, the biggest number among the four counted values is considered as the default number of the present substances. A mathematical formula was applied to guess all possible matches according to a data table of R_f profiles of standards already calculated by the same method. The validation parameters obtained in LLE were

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linearly range of 50-1000 $\mu\text{g mL}^{-1}$ biological fluid ($r \geq 0.9815$). This method has shown its suitable applicability in order to rapidly identify a wide verity of substances of toxicological interest present in the biological samples. Moreover, it's inexpensive and could be suggested in various routine drug screening processes, especially for toxicological/forensic analysis.

Keywords: Thin layer chromatography, multiple screening systems, toxicology, LLE, R_f

1. Introduction

Thin layer chromatography or planar chromatography is a chromatographic technique commonly used to separate components for analytical (analytical TLC) or purification (preparative TLC) purpose [10]. It consists of:

- A stationary phase: a thin layer of adsorbent material [10] (usually silica gel, aluminum oxide or cellulose) of 100-200 mm generally deposited on a rectangular plate of glass, aluminum, of few centimeters in dimensions. There are 3 steps to perform before the analysis [8]:
- Preparation of plates
- Sample deposit
- Plate development

The plate development is performed in specific solutions called liquid phases [4] [5]. A liquid phase, also called mobile phase or eluent is a solvent or a mixture of solvents which leads to the separation of the analytes along the stationary phase. Generally, the adsorption phenomenon is predominant (but it is also shared if the solvent is a mixture).

Multi-system thin-layer chromatography is carried out on 4 mobile phase systems in sufficiently saturated closed phases in the twin-through development chambers TA, TD, TE, TB [1].

Thin layer chromatography was by far the most commonly used separation technique for the characterization and the isolation of numerous organic and inorganic compounds (Cooper & Negrusz, 28 May 2013). Many toxicology laboratories still use this technique and its variants in order to perform preliminary drug screening routine processes which could be of an important value in terms of drug analysis orientation. Moreover, TLC spots could be exploited for a further qualitative analysis using colored specific reagents [9] [11] and /or semi-quantitative analysis by applying advanced quantification technics such as NMR Spectroscopy [2] and HPLC on the separated spots after special pretreatments [6].

Several methods have been described in the literature for the drugs toxicological screening and qualitative study by TLC, the majority of the papers describe methods whose qualification is based exclusively on specific color reactions performed on the spots.

Since TLC assays are cheap and cost effective and widely used by laboratories with limited assets, it has been considered as a good choice for the qualitative analysis of many compounds. In addition, LLE is still among the most employed techniques for sample preparation in routine analysis [7]. The purpose of this investigation was to develop a simple analytical method alongside to the LLE extraction and TLC for the identification of substances of toxicological interest in urine, bile and gastric contents.

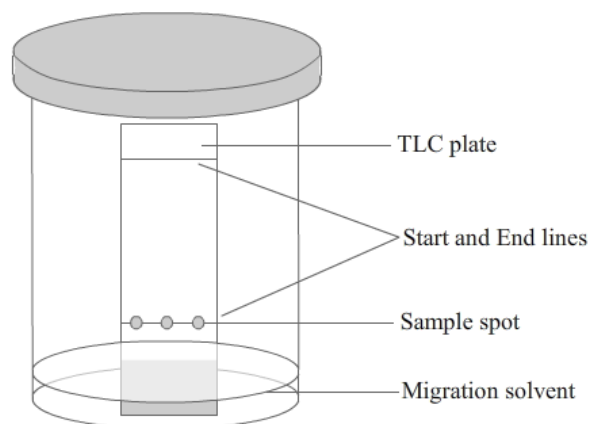


Fig. 1. TLC development chamber.

2. Experiment

2.1. Standard solutions and chemicals

The standards were all obtained from Sigma-Aldrich® (Spain). Stock standard solutions were prepared by dissolution of each drug in methanol in order to obtain a concentration of 1 mg mL⁻¹. Dilution series were prepared from the standard solutions in the following concentrations: 30, 50, 75, 100, 300, 600 and 1000 ng mL⁻¹. All these solutions were stored at -20°C in the absence of light.

TA mobile phase was prepared by mixing methanol:ammoniac 5% (50:0.750, v/v). TD mobile phase was prepared by mixing chloroform:acetone (40:10, v/v). TE mobile phase was prepared by mixing Ethyl acetate:methanol:ammoniac (42.3:5:2.5, v/v/v). TB mobile phase was prepared by mixing cyclohexan:toluene:diethylamine (37.5:7.5:5, v/v/v). The development chambers were closed and saturated for 30 minutes. The TLC plates were desiccated in the oven at 100°C for 30 minutes.

Methanol, ammoniac, chloroform, acetone, Ethyl acetate, cyclohexane, toluene, diethyl-amine were purchased from Sigma-Aldrich® (Spain). All the chemicals were of analytical grade in the highest purity available. Water was distilled and purified using a Millipore® distillation system (USA).

2.2. Biological samples

Urine, bile, and gastric contents samples were collected from healthy and clean subjects in CMBL® plastic specimen cup 60 mL. The volunteers were given written consent to participate in the investigation.

2.3. Instrumentation and analytical conditions

The analyses were carried out using Merk® TLC pre-coated silica gel plates with green fluorescent indicator 60F₂₅₄ (Switzerland) and a Spectroline® E-series UV lamp (Sigma Aldrich USA).

The 5 µL Camag micropipettes were used to apply the solutions to the plates.

2.4. Liquid-liquid extraction procedure

Aliquots of 10 mL of urine, bile and filtered gastric contents spiked with 500 µL of each group of eight standard solutions and 100 µL of reference solution (Paracetamol 3 µg mL⁻¹, Amitriptyline 3 µg mL⁻¹, Prazepam 3 µg mL⁻¹, Trimipramine 3 µg mL⁻¹) were double-extracted. The first extraction was performed after acidification by 1 to 2 drops of HCl 6 N pH 2 with 10 mL of chloroform whereas the second extraction was performed after alkalization by 1 to 2 drops of NaOH 10 N pH 9.5 with 10 mL of dichloromethane. The organic phases were dehydrated by anhydrous sodium sulfate filtered and transferred to conical tubes where they were evaporated at 65°C. The residues were dissolved in 500 µL of methanol, mixed and spotted onto the TLC plates.

2.5. Method validation

The proposed method was validated by specificity, accuracy, precision, and robustness according to the ICH guidelines and the guidelines described by Ferenczi-Fodor et al.

2.5.1. Specificity

Specificity of the method was checked by TLC of working standard solutions of 25 molecules and sample solutions spiked by those molecules. Specificity was determined by applying appropriate chromatographic conditions (such as proper mobile phase). To estimate the specificity of developed method, comparison of chromatographic bands was made.

2.5.2. Accuracy

The accuracy of the method was evaluated by measurement of recovery. Percent recovery was performed using the standard addition method. For this purpose, known amounts of reference standard of each molecule were added to the samples (urine, bile, gastric contents) in given quantities: 50%, 100%, 150% level of test concentrations (10.00, 7.50 and 5.00 per 5 mL). This analysis was performed four times. The percentage of recovery of all drug components was calculated.

2.5.3. Precision

Repeatability (intraday precision) of this method was determined by the analysis of three replicates of sample solutions at three different concentrations of each molecule (10.00, 7.50, and 5.00 per 5 mL of solution). All solutions were prepared independently and repeated three times. 5 μ L of prepared solutions was used in each case. Precision was determined on the basis of densitometric measurements of obtained spots as the relative standard deviation (coefficient of variation: CV [%]).

2.5.4. Robustness

The robustness was evaluated during the development of the method by making changes to parameters. Robustness test was prepared according to guidelines described in the papers by

Ferenczi-Fodor et al. and Nagy-Turák et al. 7.50 and 1.50 μ g \cdot spot⁻¹ of each molecule were spotted on the plates that were next developed after altering the conditions. The conditions changed were the chamber type (10 cm \times 10 cm, 20 cm \times 20 cm), the plate dimensions (10 cm \times 10 cm, 20 cm \times 20 cm, 8 cm \times 10 cm) the temperature of plate activation (\pm 15°C), the total distance of development (\pm 0.5 cm), saturation time of the chamber (\pm 10 min), the volume of chloroform (\pm 0.1 mL), methanol (\pm 0.1 mL), ammoniac (\pm 0.1 mL), acetone (\pm 0.1 mL), toluene (\pm 0.1 mL), cyclohexane (\pm 0.1 mL), and the volume of diethylamine (\pm 0.1 mL) in used mobile phase. The main effects of five factors were tested on two levels in eight experiments.

Moreover, 3 experiments were conducted on 24 randomly chosen molecules by three different manipulators in different days. (Table 1).

Table 1. Robustness additional evaluation method by performing 3 different experiments by 3 different manipulators in 3 different days with the same method parameters. (*) No spot was detected.

		R_{Fs} in TA			R_{Fs} in TD			R_{Fs} in TE			R_{Fs} in TB		
Molecules/Experiments		Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp3
Standard molecules	Amitriptyline	0,50	0,57	0,60	-*	-	-	0,80	0,82	0,78	0,77	0,69	0,80
	Paracetamol	0,80	0,81	0,75	0,25	0,22	0,25	0,63	0,66	0,69	-	-	-
	Prazepam	0,80	0,85	0,79	0,70	0,66	0,72	0,85	0,88	0,90	0,04	0,07	0,05
	Trimipramine	0,66	0,69	0,72	-	-	-	0,90	0,88	0,89	0,87	0,84	0,84
1	INH	0,69	0,66	0,71	-	-	-	0,38	0,39	0,45	-	-	-
2	Chlorpromazine	0,56	0,54	0,58	-	-	-	0,81	0,88	0,94	0,69	0,68	0,71
3	Prednisone	0,85	0,90	0,88	0,25	0,26	0,29	0,56	0,70	0,69	-	-	-
4	Paroxetine	0,25	0,28	0,29	-	-	-	0,44	0,44	0,48	0,10	0,10	0,10
5	Diclofenac	0,88	0,85	0,83	0,42	0,45	0,47	0,13	0,19	0,21	-	-	-
6	Mianserine	0,69	0,70	0,71	0,15	0,16	0,18	0,81	0,88	0,94	0,60	0,56	0,64
7	Bromazepam	0,90	0,81	0,84	0,13	0,11	0,19	0,75	0,87	0,86	0,06	0,04	0,06
8	Olanzapine	0,63	0,64	0,68	0,80	0,88	0,83	0,67	0,69	0,65	0,20	0,13	0,18
9	Clomipramine	0,69	0,50	0,54	0,04	0,03	0,03	0,83	0,79	0,80	0,79	0,82	0,80
10	Haloperidol	0,76	0,73	0,75	-	-	-	0,88	0,85	0,80	0,25	0,20	0,20
11	Sulpiride	0,40	0,38	0,44	-	-	-	0,43	0,40	0,39	-	-	-
12	Aspirine	0,83	0,78	0,78	0,16	0,20	0,14	0,10	0,09	0,09	-	-	-
13	Acebutolol	0,47	0,39	0,45	-	-	-	0,37	0,23	0,35	-	-	-
14	Tetrazepam	0,84	0,78	0,79	0,71	0,66	0,69	0,87	0,79	0,84	0,40	0,32	0,34
15	Atarax	0,81	0,75	0,76	-	-	0,00	0,69	0,58	0,66	0,21	0,12	0,10

16	Carbamazepine	0,81	0,75	0,76	0,26	0,33	0,41	0,68	0,59	0,66	0,04	0,05	0,03
17	Lamotrigine	0,73	0,76	0,74	-	-	-	0,54	0,62	0,59	-	-	-
18	Diazepam	0,76	0,78	0,80	0,71	0,65	0,66	0,81	0,86	0,80	0,35	0,38	0,30
19	Furosemide	0,80	0,79	0,80	0,16	0,10	0,13	0,10	0,06	0,10	0,00	0,00	0,00
20	Levopromazine	0,59	0,62	0,60	-	-	-	0,73	0,78	0,76	0,69	0,74	0,70
21	Phenobarbital	0,74	0,75	0,73	0,61	0,62	0,64	0,75	0,78	0,70	-	-	-

Table 2. R_{fs} profiles of the 25 molecules with standard deviations values $\sigma_{A, D, F, B}$ calculated using equation (1).

Molecules/Experiments		R_{fs} in TA		R_{fs} in TD		R_{fs} in TE		R_{fs} in TB	
		Mean	σ_A	Mean	σ_D	Mean	σ_E	Mean	σ_B
Standard molecules	Amitriptyline	0,56	0,05	-	-	0,80	0,02	0,75	0,06
	Paracetamol	0,79	0,03	0,24	0,02	0,66	0,03	-	-
	Prazepam	0,81	0,03	0,69	0,03	0,88	0,03	0,05	0,02
	Trimipramine	0,69	0,03	-	-	0,89	0,01	0,85	0,02
1	INH	0,69	0,03	-	-	0,41	0,04	-	-
2	Chlorpromazine	0,56	0,02	-	-	0,88	0,07	0,69	0,02
3	Prednisone	0,88	0,03	0,27	0,02	0,65	0,08	-	-
4	Paroxetine	0,27	0,02	-	-	0,45	0,02	0,10	0,00
5	Diclofenac	0,85	0,03	0,45	0,05	0,18	0,04	-	-
6	Mianserine	0,70	0,01	0,16	0,02	0,88	0,07	0,60	0,04
7	Bromazepam	0,85	0,05	0,14	0,04	0,83	0,07	0,05	0,01
8	Olanzapine	0,65	0,03	0,84	0,04	0,67	0,02	0,17	0,04
9	Clomipramine	0,58	0,10	0,03	0,01	0,81	0,02	0,80	0,02
10	Haloperidol	0,75	0,02	-	-	0,84	0,04	0,22	0,03
11	Sulpiride	0,41	0,03	-	-	0,41	0,02	-	-
12	Aspirine	0,80	0,03	0,17	0,03	0,09	0,01	-	-
13	Acebutolol	0,44	0,04	-	-	0,32	0,08	-	-
14	Tetrazepam	0,80	0,03	0,69	0,03	0,83	0,04	0,35	0,04
15	Atarax	0,77	0,03	-	-	0,64	0,06	0,14	0,06
16	Carbamazepine	0,77	0,03	0,33	0,08	0,64	0,05	0,04	0,01
17	Lamotrigine	0,74	0,02	-	-	0,58	0,04	-	-
18	Diazepam	0,78	0,02	0,67	0,03	0,82	0,03	0,34	0,04
19	Furosemide	0,80	0,01	0,13	0,03	0,09	0,02	-	-
20	Levopromazine	0,60	0,02	-	-	0,76	0,03	0,71	0,03
21	Phenobarbital	0,74	0,01	0,62	0,02	0,74	0,04	-	-

3. Results and Discussion

The methods of routine drug screening must show high robustness, compatibility with multiple biological samples, and be low cost. Drug identification with TLC in multiple screening systems is the most effective and reliable method in this case.

3.1. Chromatographic conditions

Several chromatographic conditions, such as mobile phases composition, plates dimensions, plate activation temperature, chambers type, development distance in order to obtain a satisfactory chromatographic separation (optimum resolution and efficiency).

Various solvents or mixture of solvents at different compositions were used to perform better extractions using LLE.

3.2. Method validation

3.2.1. Specificity

Mixtures as described above, TA methanol:ammoniac 5% (50:0.750, v/v). TD chloroform:acetone (40:10, v/v). TE Ethyl acetate:methanol:ammoniac (42.3:5:2.5, v/v/v). TB cyclohexan:toluene:diethylamine (37.5:7.5:5, v/v/v) were chosen for development as optimum mobile phases, because they enabled –combined–successful separation of the 25 molecules.

3.2.2. Accuracy

Accuracy of this TLC method was evaluated by measuring recovery. The average recovery was generally equal to 97.3%, 98.7%, 98% for 50%,

100%, and 150% amount of standard of each molecule.

3.2.3. Precision

Precision of the method was investigated by measurement by densitometry of each spot obtained on the basis of sample solutions prepared at three different known concentrations of each molecule. This analysis was reinforced by HPLC-DAD/UV analysis of obtained spots.

3.2.4. Robustness

As previously described, various small deliberate changes were made to the chromatographic parameters, the main five factors were tested on two levels in eight experiments. It was concluded that none of these factors had significant effect on the results.

In addition, 3 different experiments were performed by 3 different manipulators with the same method parameters. The measured R_F were very close with a standard deviation of ($\pm 0,07$) (Table 1).

3.2.5. Computational identification of molecules from their R_F profiles

A simple database was built in MySQL that allowed to store and/or to update R_F profiles. A small web-based software was written in PHP that allowed to compare found R_F with those stored in the database and predict all match probabilities. The program allowed to store as many molecule-profile data as desired (**Table 2**).

4. Equations

The standard variation of the calculated R_f for each molecule is given as follow:

$$s = \sqrt{\frac{\sum_{i=1}^N (x_i - \bar{x})^2}{N - 1}} \quad (1)$$

Where $\{x_1, x_2, x_3, \dots, x_N\}$ are the observed values of R_f during each assay. \bar{x} is the mean value of these observations. And N the number of observations for each molecule.

5. Conclusions

A suitable TLC technique in multiple screening systems was developed and validated using LLE as extraction procedure for the identification of 25 drugs in human urine, bile, and gastric contents. This method provides a good alternative to classic mono-system TLC for drugs screening and identification. The major advantage of this method is that it is Robust and is performed in isocratic mode. Moreover, it is relatively cost effective and can be used as a daily reliable routine technique for drug screening in human urine, bile, and gastric contents.

Using HPLC-DAD/UV as a complementary technique would refine search list and provide more precision in terms of drug identification.

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