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Short communication

Qualitative screening for basic drugs in autopsy liver samples by dual-plate overpressured layer chromatography

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Abstract

An overpressured layer chromatography (OPLC) method was evaluated for broad-scale screening of basic drugs in 5 g autopsy liver samples using two parallel OPLC systems. Sample preparation included enzymatic digestion with trypsin and liquid–liquid extraction with butyl chloride. Chromatographic separation was performed as dual-plate analysis, with mobile phases composed of trichloroethylene–methylethylketone–*n*-butanol–acetic acid–water (17:8:25:6:4, v/v) (OPLC1), and butyl acetate–ethanol (96.1%)–tripropylamine–water (85:9.25:5:0.75, v/v). Identification was based on automated comparison of corrected R_f values (hR$_f$c) and *in situ* UV spectra with library values by dedicated software. The identification limit was determined for 25 basic drugs in liver ranging from 0.5 to 10 mg/kg. The OPLC method proved to be well suited for routine screening analysis of basic drugs in post-mortem samples of varying quality, combining the benefit from moderately high separation power with the ease of disposable plates.

Keywords: Drug screening; Tissue; Planar chromatography; OPLC

1. Introduction

Qualitative screening analysis for drugs and poisons is a key step in post-mortem forensic toxicological investigations. For a thorough investigation, the screening method should be capable of detecting and identifying hundreds of exogenous compounds in biological material. Urine is the primary specimen for qualitative analysis, but in cases where urine is not available, liver samples are a good choice for basic lipophilic drugs. However, considering the severe matrix effects caused by tissue background, the standard screening methods based on gas chromatography and liquid chromatography may not be optimally suited for analysing liver samples.

Instrumental thin-layer chromatography (TLC), utilizing corrected R_f values (hR$_f$c) [1] and *in situ* UV spectra as identification parameters, has been successfully used in broad-scale drug screening [2–4]. Because of the use of disposable plates, TLC is particularly tolerant of fatty extracts that may ruin the performance of column chromatography. The limited separation power of free-flow TLC is, however, a restricting factor in utilisation of the technique.

In overpressured layer chromatography (OPLC), the technique originally developed by Tyihák and Mincsovics [5], the separation is performed in a pressurized chamber and the flow rate of the mobile phase is kept constant by an external pump. In OPLC, longer separation distances and consequently higher spot numbers are achieved than in TLC, because diffusion is diminished [6,7]. Being a planar chromatographic technique OPLC provides an ideal platform for analysing post-mortem tissues of varying quality. An overview of the technique and its applications has been given by Nyiredy [8].

Enzyme digestion combined with liquid–liquid extraction has been the method of choice for tissue samples instead of mechanical homogenisation in many analysis methods for basic drugs [9–11]. The liquid–liquid extraction procedures have involved either a single extraction at basic pH, or a basic extraction with an additional back extraction step for improved purity.

Only a few papers describe the use of OPLC in the forensic setting [12,13]. A broad-scale drug screening system for urine samples based on OPLC with two independent separation systems has been described and validated earlier [6,14]. In this

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paper, the approach is adopted for liver analysis following an efficient sample work-up procedure.

2. Experimental

2.1. Materials and apparatus

The chromatographic plates were 20 cm × 20 cm aluminum sheets coated with a 150–160 μm layer of silica gel F_{254} of 5-μm particle size. The plates were original Merck HPTLC (Darmstadt, Germany) products, modified for OPLC use by OPLC-NIT (Budapest, Hungary). Drug standards were obtained from various pharmaceutical companies and were of pharmaceutical purity. Standard stock solutions and correction standard mixtures were prepared in methanol at a concentration of 1 or 2 mg/mL, and dilutions were prepared in methanol as needed. All solvents used were of analytical reagent grade. Fast Black K salt (FBK) was from Aldrich (Milwaukee, WI, USA). Trypsin type IX-S was from Sigma (Steinheim, Germany). Liver samples were collected at autopsies.

Sample application to the plates was performed with an automatic TLC sampler ATS III (Camag, Muttenz, Switzerland). The OPLC instrument was a Personal OPLC Basic System 50 (OPLC-NIT). A TLC Scanner 3 (Camag) operated with winCats 1.2.6 software was used for scanning densitometry.

2.2. Sample preparation

Liver samples of 5 g were cut into 5 mm × 5 mm pieces with scissors and 8 mL of 0.1 M Sorensen phosphate buffer (pH 7.5) was added together with 1 mL of 5 mg/mL trypsin solution and a few glass beads. The samples were digested in a shaking water bath for 2 h in 57 °C and filtered through a nylon net. The pH was adjusted to 2.5–3.0 with 5 M hydrochloric acid and the samples were extracted with 10 mL of diethyl ether on a bench top shaker for 10 min. After centrifugation for 5 min at 5000 rpm the organic phase was removed and discarded. The pH of the remaining aqueous phase was adjusted to 11–12 with 5 M sodium hydroxide, and the liquid was extracted with 10 mL of butyl chloride. After centrifugation, the organic phase was back extracted with 3 mL of 0.05 M sulphuric acid. The aqueous phase was made alkaline with 1 M tris(hydroxymethyl) aminomethane buffer, and extracted again with 10 mL of butyl chloride. The organic phase was evaporated to dryness at 40 °C under air, and the residue was reconstituted with 100 μL of methanol. The extract was vortex mixed and centrifuged, and the clear supernatant was used for the analysis. An aliquot of 10 μL was applied to each of the two OPLC plates as a narrow band with ATS III using the spray mode.

2.3. Planar chromatography and detection

For OPLC1, the correction standard mixture components were codeine ($hR_{f}c = 16$), promazine ($hR_{f}c = 38$), nortriptyline ($hR_{f}c = 58$), moperone ($hR_{f}c = 76$), and theophylline ($hR_{f}c = 86$). The amount of each component applied to the plate was 1 μg. The mobile-phase composition was trichloroethylene–methylethylketone–*n*-butanol–acetic acid–water (17: 8:25:6:4, v/v), and the plate was developed without presaturation. The external pressure was 50 bar, the flow rate 450 μL/min, the volume of rapid delivery 300 μL, and the mobile-phase volume 5500 μL (development time 12 min 19 s) [6].

For OPLC2, the correction standard mixture components were codeine ($hR_{f}c = 9$), promazine ($hR_{f}c = 19$), amitriptyline ($hR_{f}c = 40$), levomepromazine ($hR_{f}c = 60$), and dextropropoxyphene ($hR_{f}c = 94$). The amount of each component applied to the plate was 1 μg. The mobile-phase composition was butyl acetate–ethanol (96.1%)–tripropylamine–water (85:9.25:5:0.75, v/v), and the plate was saturated with the mobile phase in a twin trough glass tank for 0.5 h immediately prior to development. The external pressure was 50 bar, the flow rate 450 μL/min, the volume of rapid delivery 300 μL, and the mobile-phase volume 5000 μL (development time 11 min 12 s) [6].

The developed plates were dried in a stream of warm air, scanned at 220 nm, and the *in situ* UV-spectra of detected spots were measured between 190 and 400 nm. The data analysis and reporting was performed automatically with the winCATS software with the additional SpeclibTool (Camag). For visualisation, the OPLC1 plate was sprayed with 0.5% aqueous solution of FBK, dried briefly in a stream of warm air, and further sprayed with 0.5 M sodium hydroxide solution. Amines react with FBK to yield orange, red, violet, and brown products, depending on the structure of the analyte [15]. The OPLC2 plate was sprayed with Salkowski reagent (60% sulphuric acid containing 0.5% iron(III)chloride) and illuminated under 366 nm UV light for 15 min. After documentation of fluorescent and coloured products, the tracks were treated with Marquis reagent (Mq, 2 drops of 40% formaldehyde solution added with a pasteur pipette to 4 mL of concentrated sulphuric acid), and the additional coloured products again documented.

2.4. Determination of identification limits

The identification limit was determined for 25 toxicologically relevant drugs. Drug free liver material was spiked with drugs after hydrolysis of the tissue. The initial concentration was 1 mg/kg, and the concentration was lowered or increased as necessary. Three replicates were performed at the identification limit level. The identification limit was determined by both instrumental analysis and visualisation if possible.

3. Results and discussion

Table 1 shows the identification limit for 25 basic drugs in liver. Term identification limit has been used here as defined by Vogelgesang and Hädrich as the lowest concentration that could safely be detected [16]. Analysis by $hR_{f}c$/UV libraries was based on an $hR_{f}c$ presearch using a detection window of ±7 $hR_{f}c$ units, followed by search by spectral correlation. Visualisation reactions were applied for confirmation. The identification limit by UV scanning densitometry was defined as the lowest concentration resulting in positive identification, generally producing the number one hit by UV spectral comparison in both sys-

Table 1
Identification limits of 25 basic drugs in liver by OPLC with UV scanning densitometry or visualisation

Drug	Identification limit in liver by UV spectral comparison (mg/kg)	Identification limit in liver by visualisation (mg/kg)[a]
Amitriptyline	2.5	1.0 (Mq[a] brown)
Chloroquine	1.0	Native violet fluorescence when wet
Chlorpromazine	5.0	0.5 (Salkowski, Mq aniline red)
Citalopram	1.0	nd[b]
Clomipramine	1.0	0.5 (Salkowski light blue)
Codeine	5.0	5.0 (Mq dark violet)
Dextropropoxyphene	7.5	2.5 (Mq dark gray)
Diltiazem	2.5	nd
Doxepin	2.5	1.0 (Mq brown)
Fluoxetine	2.5	1.0 (FBK[a])
Fluvoxamine	5.0	nd
Levomepromazine	1.0	1.0 (Salkowski, Mq violet)
Melperone	2.5	nd
Mianserin	2.5	nd
Mirtazapine	1.0	nd
Moclobemide	1.0	nd
Nortriptyline	2.5	1.0 (FBK, Mq orange-brown)
Olanzapine	2.5	2.5 (FBK)
Orphenadrine	5.0	1.0 (Mq yellow)
Paroxetine	5.0	2.5 (FBK, Mq greenish)
Propranolol	1.0	1.0 (FBK)
Quinine	2.5	0.5 (Salkowski + 15 min UV (366 nm) blue fluoresc.)
Thioridazine	10.0	1.0 (Salkowski, Mq turquoise)
Tramadol	2.5	2.5 (Mq brownish)
Verapamil	2.5	nd

[a] Mq: Marquis reagent; FBK: Fast Black K reagent, for preparation of the visualisation reagents see Section 2.
[b] nd: not detected.

tems. Based on densitometry the median identification limit was 2.5 mg/kg, ranging from 1.0 to 10.0 mg/kg. Based on visualisation reactions, the median identification limit was 1.0 mg/kg, ranging from 0.5 to 5.0 mg/kg. In most cases where a visualisation reaction was applicable the identification limit was lower by visualisation than by UV, which is a unique advantage of planar chromatography.

The extraction procedure was preceded by a pre-extraction with diethyl ether in acidic conditions, effectively removing fat and acidic/neutral compounds while the basic analytes remained in the aqueous phase. The subsequent liquid–liquid extraction procedure with butyl chloride is cost-effective and produces clean extracts in spite of the occasionally fatty samples (Fig. 1).

Identification limit or detection limit values have been rarely given in the literature for tissue samples in the forensic toxicology context. Huang et al. reported that a selection of acidic, neutral and basic drugs could be identified and quantified at the level 10 mg/kg by GC analysis, but exact detection limits were not reported [17]. Heinig and Bucheli reported detection limits of 2–10 ng/mL tissue homogenate for various types of analytes by LC/MS/MS with column switching [18]. The identification limits of the current method are obviously higher than in an LC/MS/MS method. However, drug concentrations in tissues are rather in tens of mg/kg than in sub mg/kg level in fatal poisonings. In a review by Musshof et al. on concentrations in fatal poisonings the amitriptyline concentration in liver varied from 0.2 to 340 mg/kg (53 cases) with a median of 40 mg/kg, and the dextropropoxyphene concentrations from 4.1 to 392 mg/kg (68 cases), with a median of 44 mg/kg [19]. In this setting, the current method is well suited for the qualitative screening analysis of basic drugs in liver samples. The fully automated data analysis of the densitometric measurements allows operation and documentation under strict quality requirements. Visualisation reactions improve the reliability of identification further. The method has been used in routine analysis in the authors' laboratory since 2004, and the method was accredited by The Finnish Accreditation Service (FINAS), the national accreditation body, in 2006. The number of cases studied by the presented method

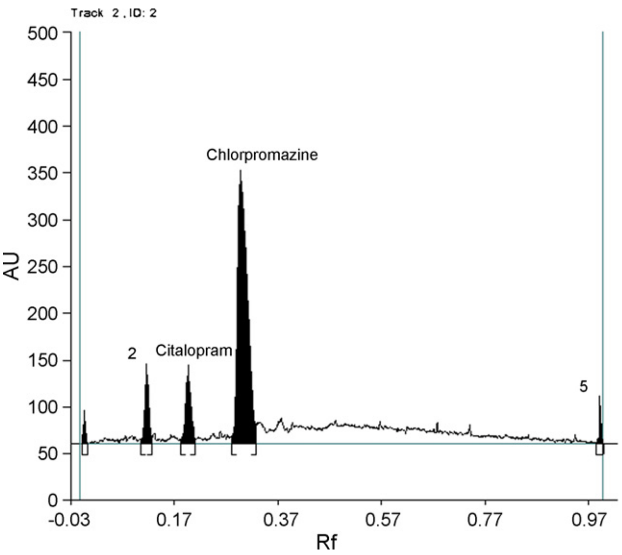

Fig. 1. OPLC densitogram of a spiked liver sample containing citalopram (1 mg/kg) and chlorpromazine (5 mg/kg). The extraction procedure resulted in clean extracts with a low background.

Table 2
Total number of findings in 1553 liver samples analysed

Drug	Total number of findings
Citalopram	86
Norcitalopram	74
Levomepromazine	55
Mirtazapine	46
Norlevomepromazine	46
Amitriptyline	40
Nortriptyline	38
Metoclopramide	34
Olanzapine	33
Propranolol	32
Cetirizine	32
Amiodarone	28
Tramadol	26
Quinine/quinidine	24
Quetiapine	21
Carbamazepine	19
Venlafaxine	17
Hydroxyzine	16
Lidocaine	16
Metoprolol	16
Doxepin	13
Haloperidol	13
Caffeine	13
Nordoxepin	12
Sertraline	12
Diltiazem	11
Fluoxetine	9
Chlorprothixene	9
Mianserin	8
Norverapamil	8
Clozapine	7
Norchlorprothixene	7
Normianserin	7
Zuclopenthixol	7
Bisoprolol	6
Chloroquine	6
Lamotrigine	6
Orfenadrine	6
Hydroxychloroquine	5
Chlorpromazine	5
Zolpidem	5
Verapamil	5
Flecainide	4
Codeine	4
Nororfenadrine	4
Oxazepam	4
Paroxetine	4
Dixyrazine	3
Duloxetine	3
Fluvoxamine	3
Carvedilol	3
Temazepam	3
Trimipramine	3
Amlodipine	2
Dextropropoxyphene	2
Nordiltiazem	2
Diphenhydramine	2
Dipyridamole	2
Ketamine	2
Melperone	2
Nordextropropoxypene	2
Nortrimipramine	2
Promazine	2
Tizanidine	2
O-Desmethyltramadol	2
Zopiclone	2
Alprazolam	1
Aminophenazone	1
Aripiprazole	1
Doxapram	1
EDDP	1
Clobutinole	1
Clomipramine	1
Methadone	1
Norclomipramine	1
Nortramadol	1
Oxycodone	1
Perphenazine	1
Promethazine	1
Ropivacaine	1
Sildenafil	1
Ziprasidone	1

was 1553 in 2006. As a result 82 different analytes were identified, and the total number of findings was 959 (Table 2). The most common findings were lipophilic basic analytes, which are more readily detected in liver samples than in urine [20]. In addition, some common acid/neutral drugs appeared to survive the extraction procedure, namely carbamazepin, temazepam and oxazepam.

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