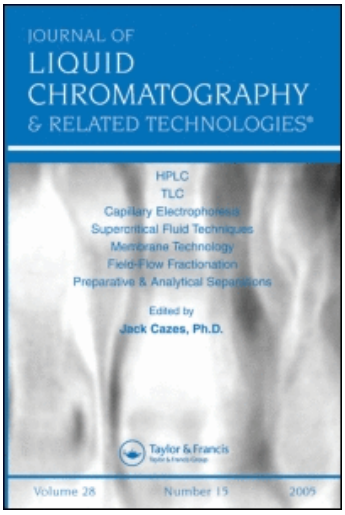

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IMPROVED IDENTIFICATION BY IN SITU UV SPECTRA IN PLANAR CHROMATOGRAPHY

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ABSTRACT

Novel software was introduced for library searching in planar chromatography, based on corrected R_f values (hR_f^c), *in situ* ultraviolet (UV) spectral correlation, and spectrum maximum site comparison. Analysis of autopsy urine samples by thin-layer chromatography (TLC) and overpressured layer chromatography (OPLC) revealed that the new method was superior to the standard method using hR_f^c and correlation only. The advantage was more pronounced in TLC, which possessed poorer separation power and generally lower quality spectra than OPLC. In 11 cases of 47 in TLC, the hit list position of the correct finding was improved by the new method.

This result indicates that the spectrum maximum site function gives a different kind of discrimination than would be obtained by only setting the correlation cut-off at a higher value. In OPLC, the hit lists were originally shorter, but an improvement in the list

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lengths was evident. OPLC proved to be the planar method of choice in the screening analysis of urine samples for drugs due to its high separation power and speed.

INTRODUCTION

Thin-layer chromatography (TLC) in the instrumental mode utilizing corrected R_f values (hR_f^c),[1] measurement of *in situ* ultraviolet (UV) spectra, and special software for identification with substance libraries, has been available for screening analysis for several years.[2-4] However, due to the limited separation power of TLC and the fairly low information content of UV spectra, long hit lists of candidates are often seen in the analysis report. In overpressured layer chromatography (OPLC), better separation numbers can be obtained compared to TLC. Recently, two complementary OPLC systems have been described for broad scale toxicological screening analysis for drugs.[5]

In spite of improved separation power, the hit lists may still remain relatively long. Use of the spectral correlation only seems to be inadequate to resolve between fairly similar UV spectra. In this study, an improved version of the identification software is described: the program uses spectrum maximum sites as an additional criterion for identification. The effect of the maximum site criterion on the length of hit lists is shown in TLC and OPLC analysis of drugs in urine.

EXPERIMENTAL

Materials and Apparatus

TLC

The chromatographic plates were 10 cm (height) x 20 cm (breadth) TLC glass plates coated with 0.25 mm layers of silica gel 60 F_{254} (Merck, Darmstadt, Germany).

OPLC

The chromatographic plates were 20 cm x 20 cm HPTLC aluminium sheets coated with 0.2 mm layers of silica gel 60 F_{254} (Merck). The plate edges were factory-sealed for OPLC use (OPLC-NIT Engineering Company, Budapest, Hungary). Drug standards were obtained from various pharmaceutical companies and were of pharmaceutical purity.

The standard and correction standard solutions were prepared in metahanol to obtain a concentration of 2 mg/mL and 1 mg/mL, respectively. Analytical grade solvents were used throughout the study. The urine samples were collected at autopsy.

The automatic TLC sampler was an ATS III (Camag, Muttenz, Switzerland). The OPLC instrument was a Personal OPLC Basic System 50 (OPLC-NIT Engineering Company). The scanning densitometer was a TLC Scanner 3 (Camag) operated with CATS 4.03 software.

Chromatography

TLC

The mobile phase composition was toluene – acetone – 94% ethanol – 25% ammonia (45:45:7:3), and the chamber was allowed to saturate with a piece of filter paper for 0.5 h before development. The correction standards were codeine ($hR_f^c = 16$), promazine ($hR_f^c = 36$), clomipramine ($hR_f^c = 49$), and cocaine ($hR_f^c = 66$).[2] The drug library consisted of 355 substances measured with 4 μg amounts of pure substances.[6]

OPLC

The mobile phase composition was trichloroethylene – methylethylketone – *n*-butanol – acetic acid – water (17:8:25:6:4), and the development was carried out without pre-saturation of the plate. The external pressure was 50 bar, the flow-rate was 450 μL/min, the volume of rapid delivery was 300 μL, and the mobile phase volume was 5500 μL. The correction standards were codeine ($hR_f^c = 16$), promazine ($hR_f^c = 38$), nortriptyline ($hR_f^c = 58$), moperone ($hR_f^c = 76$), and theophylline ($hR_f^c = 86$).[5] The drug library consisted of 113 substances measured with 1 μg amounts of pure substances.

Urine Analysis

The sample preparation for basic drugs in 5 mL urine specimens involved an ion-pair extraction with dichloromethane containing 0.01 M bis(2-ethyl-hexyl)phosphoric acid at pH 7.5.[6] Aliquots of the reconstituted extracts, 10 μL for TLC and 1 μL for OPLC, and the correction standard mixture on a separate track, were applied band-wise to the plates with the automatic TLC sampler. The developed plates were evaluated by densitometry at 220 nm, and the *in situ* UV

Table 1. Hit Lists Obtained in the TLC Analysis of Urine Samples Using a) the Novel Software with hR_f^c + Spectrum Correlation + Spectrum Maximum Site Function (Method A) and by b) hR_f^c + Spectrum Correlation Only (Method B)

Number of Case	Correct Finding	a) Hit List Position of Correct Finding/ Hit List Length	b) Hit List Position of Correct Finding/ Hit List Length
H1034	Thioridazine	1st/2	2nd/11
2480	Zopiclone	1st/1	1st/4
2491	Amitriptyline	1st/2	1st/6
2499	Zopiclone	1st/1	1st/4
2520	Dextropropoxyphene	2nd/5	4th/12
2540	Levomepromazine	2nd/5	2nd/21
2553	Citalopram	1st/5	1st/24
2599	Quinine/Quinidine	1st/1	1st/3
2601	Quinine/Quinidine	1st/1	1st/4
2634	Citalopram	1st/4	1st/17
2647	Citalopram	1st/6	1st/18
2662	Nordoxepin	1st/3	1st/24
2674	Thioridazine	1st/2	1st/13
2701	Levomepromazine	2nd/3	2nd/17
2716	Citalopram	1st/1	4th/28
2759	Levomepromazine	2nd/5	2nd/11
2761	Quinine/Quinidine	1st/1	1st/4
2771	Orphenadrine	4th/11	5th/17
2784	Zopiclone	1st/3	1st/4
2801	Promazine	1st/6	2nd/20
2816	Citalopram	2nd/6	2nd/24
2823	Promazine	1st/7	3rd/27
2869	Orphenadrine	3rd/7	3rd/18
2889	Atenolol	1st/5	1st/15
2906	Metoclopramide	1st/3	1st/7
2931	Citalopram	1st/5	1st/18
2936	Promazine	2nd/8	2nd/22
2948	Amitriptyline	1st/1	1st/30
2948	Nortriptyline	1st/1	1st/19
2965	Metoprolol	4th/10	4th/20
3026	Sotalol	1st/6	1st/16
3054	Promazine	1st/7	2nd/20
3063	Zopiclone	1st/1	2nd/4
3085	Amphetamine	2nd/6	2nd/12
3098	Nordoxepin	1st/2	1st/19
3101	Doxepin	1st/1	1st/30
3109	Quinine/Quinidine	1st/1	1st/2

Table 1. Continued

Number of Case	Correct Finding	a) Hit List Position of Correct Finding/ Hit List Length	b) Hit List Position of Correct Finding/ Hit List Length
3132	Metoclopramide	1st/1	2nd/3
3134	Metformin	1st/1	1st/12
3134	Metoprolol	5th/11	5th/18
3193	Metoprolol	2nd/7	2nd/12
3218	Citalopram	1st/5	1st/13
3239	Ranitidine	1st/1	1st/5
3242	Zopiclone	1st/2	1st/4
3250	Promazine	1st/3	3rd/14
3252	Metoprolol	4th/5	5th/6
3272	Promazine	1st/6	1st/20

spectra were measured using a spectral range of 190-400 nm with 5 nm wavelength increments. CATS Spectrum Library for MS Windows (Camag) and a modified version of the software, developed in the present study, was used for the correction of R_f values and for the library search based on hR_f^c values and UV spectra.

RESULTS AND DISCUSSION

The novel software developed for substance identification in planar chromatography is an extension of the CATS Spectrum Library for MS Windows (Camag). In addition to hR_f^c and spectral correlation with substance libraries, identification within this program can also be based on the absorption maximum sites of the spectrum. It is also possible to be limited within a certain spectral region. In order to avoid too strict identification criteria, the number of maximum sites (Nmax) can be individually set at a lower number than the actual number for each library compound, and identification is based on any combination of maximum sites present in the sample spectrum.

The TLC library (355 substances) and OPLC library (133 substances) were updated by adding the spectrum maximum sites with wavelength windows to each substance individually. Based on preliminary experiments, Nmax was usually set at 1-3. For substances with one or two maximum sites in their spectrum, Nmax was given an equal value, but for substances with three or more maximum sites, Nmax was given a value of one less. The spectral region above 340 nm was

Table 2. Hit Lists Obtained in the OPLC Analysis of Urine Samples Using a) the Novel Software with hR_f^c + Spectrum Correlation + Spectrum Maximum Site Function (Method A) and by b) hR_f^c + Spectrum Correlation Only (Method B)

Number of Case	Correct Finding	a) Hit List Position of Correct Finding/ Hit List Length	b) Hit List Position of Correct Finding/ Hit List Length
2480	Zopiclone	1st/1	1st/1
2491	Amitriptyline	1st/2	1st/12
2491	Nortiptyline	1st/1	1st/6
2495	Diltiazem	1st/6	1st/10
2499	Zopiclone	1st/1	1st/1
2540	Chlorpromazine	1st/2	1st/6
2553	Citalopram	1st/4	1st/8
2599	Quinine/Quinidine	1st/1	1st/2
2634	Citalopram	1st/2	1st/3
2647	Citalopram	1st/2	1st/3
2662	Doxepin	1st/1	1st/7
2680	Citalopram	1st/2	1st/3
2680	Amitriptyline	1st/3	1st/12
2716	Zopiclone	1st/1	1st/1
2716	Citalopram	1st/6	1st/11
2716	Norcitalopram	1st/2	1st/9
2729	Chlorpromazine	1st/1	1st/2
2761	Quinine/Quinidine	1st/1	1st/1
2771	Orphenadrine	1st/7	1st/12
2784	Zopiclone	1st/1	1st/1
2816	Citalopram	1st/5	1st/8
2823	Promazine	1st/2	1st/3
2869	Norорphenadrine	1st/7	1st/17
2889	Atenolol	1st/3	1st/4
2906	Metoclopramide	1st/1	1st/2
2931	Citalopram	1st/6	1st/8
2931	Norcitalopram	1st/2	1st/8
2948	Amitriptyline	1st/2	1st/13
2948	Atenolol	1st/3	1st/4
2948	Nortriptyline	1st/2	1st/7
2950	Doxepin	1st/1	1st/3
3025	Sotalol	1st/6	1st/7
3032	Doxepin	1st/1	1st/6
3054	Promazine	1st/1	1st/5
3075	Doxepin	1st/1	1st/6
3085	Dextropropoxyphene	1st/5	1st/12
3098	Doxepin	1st/1	1st/10

Table 2. Continued

Number of Case	Correct Finding	a) Hit List Position of Correct Finding/ Hit List Length	b) Hit List Position of Correct Finding/ Hit List Length
3098	Nordoxepin	1st/1	1st/12
3101	Doxepin	1st/1	1st/9
3109	Quinine/Quinidine	1st/1	1st/1
3119	Atenolol	1st/3	1st/4
3132	Metoclopramide	1st/1	1st/2
3134	Metoprolol	1st/11	1st/14
3183	Diltiazem	1st/3	1st/6
3193	Metoprolol	1st/12	1st/14
3218	Citalopram	1st/1	1st/3
3239	Ranitidine	1st/1	1st/2
3239	Zopiclone	1st/1	1st/1
3239	Propranolol	1st/1	1st/1
3242	Zopiclone	1st/1	1st/1
3245	Amitriptyline	1st/3	1st/10
3247	Norcitalopram	1st/1	1st/2
3272	Promazine	1st/2	1st/5

rejected due to possible uncharacteristic maxima. Concentration differences and matrix effects of urine samples caused 1-3 nm shifts of the maximum sites, depending on the substance and type of spectrum, and thus a ±3 nm wavelength window was set as a constant for all maximum sites of all library compounds.

Tables 1 and 2 compare the hit lists obtained by TLC and OPLC, respectively, using the spectrum maximum site function (Method A) and the standard software (Method B) in the analysis of urine samples. In these experiments, the hR_f^c window was ± 7, and the spectrum correlation cut-off value was 0.8. The improvement was more pronounced in TLC, as could be expected on grounds of the lower separation power of the technique and the larger library size used. In 11 cases of 47 in TLC, the hit list position of the correct finding improved. This result indicates, that the spectrum maximum site function gives a different kind of discrimination than what would be obtained by only setting a higher correlation cut-off value. In OPLC, the hit lists were originally shorter, but an improvement in the list lengths was still evident. The chromatographic separation by TLC and OPLC in three selected cases is compared in Figures 1-3.

Although it has been possible to measure *in situ* UV spectra for more than thirty years,[7-9] very little literature is available on the practical use of large UV spectrum libraries in planar chromatography. It has been stated that UV library

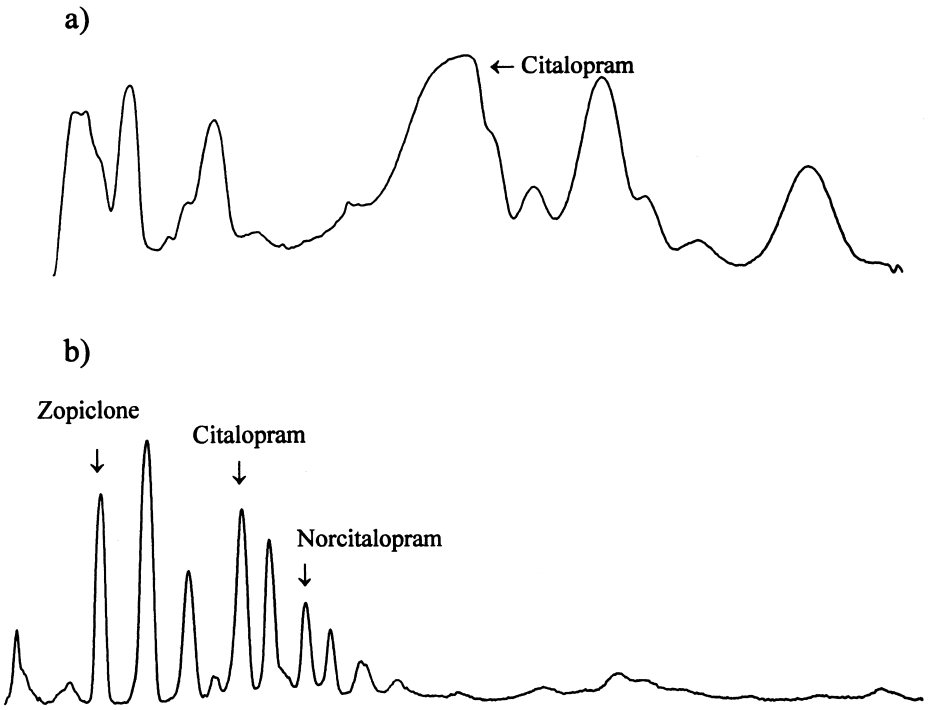

Figure 1. Chromatograms obtained from case 2716 urine sample by a) TLC with the finding citalopram, and b) OPLC with the findings zopiclone, citalopram and norcitalopram.

search procedures within planar techniques are not useful,[10] but the recent work done in the authors' laboratory[2-6] shows the opposite.

CONCLUSIONS

Novel software, which utilized both UV spectral correlation and spectrum maximum sites for identification, was found to be superior in terms of hit list length and validity of results, compared to the standard program using correlation only. This was especially pronounced in ordinary TLC, which exhibited poorer separation and generally lower quality spectra than OPLC. It is evident that OPLC possesses great potential in broad scale screening analysis for drugs due to

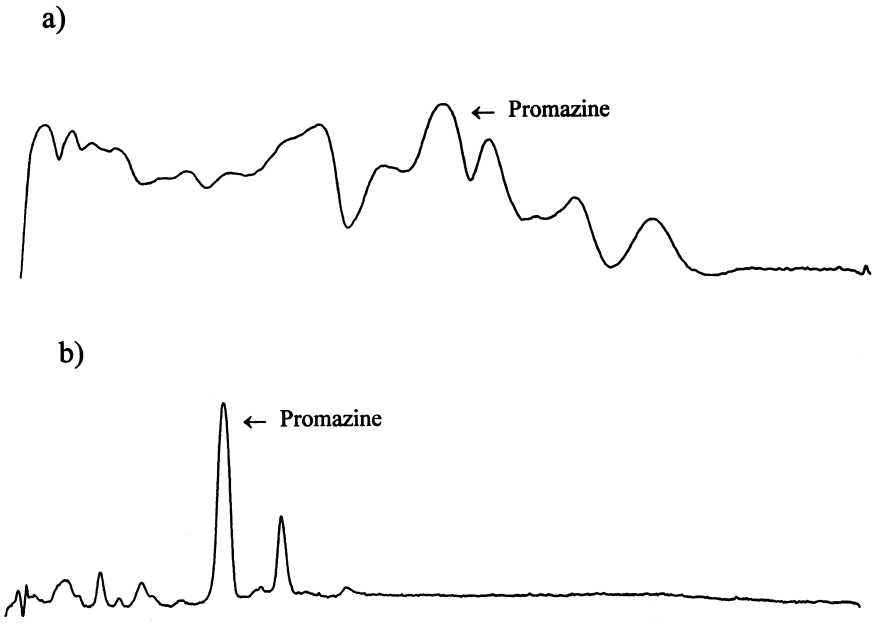

Figure 2. Chromatograms obtained from case 2823 urine sample by a) TLC and b) OPLC, with the finding promazine.

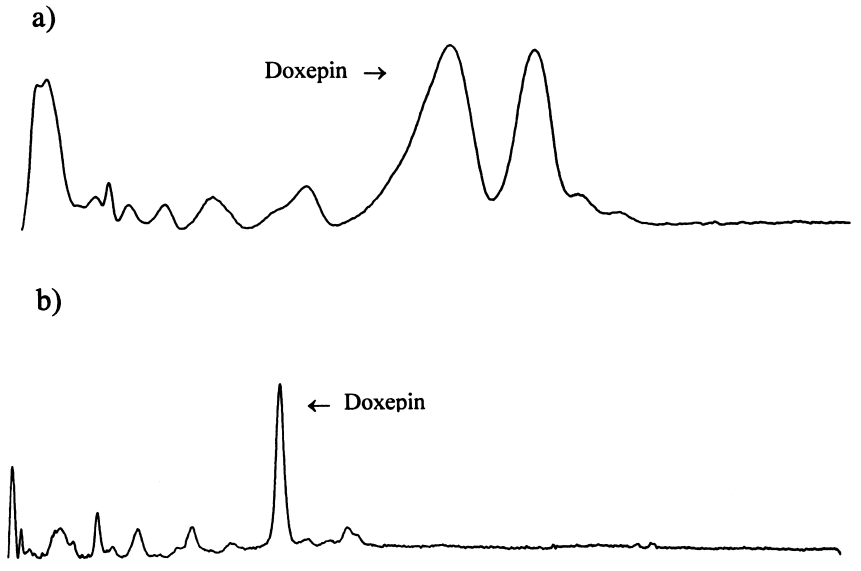

Figure 3. Chromatograms obtained from case 3101 urine sample by a) TLC and b) OPLC, with the finding doxepin.

its high separation power and speed, especially in connection with these new means of UV spectral discrimination.

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