THE COMBINATION OF TWO-DIMENSIONAL THIN LAYER CHROMATOGRAPHY AND REMISSION SPECTROMETRY – A CHROMATOGRAPHIC TECHNIQUE WITH HIGH IDENTIFICATION POWER FOR SYSTEMATIC TOXICOLOGICAL ANALYSIS

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ABSTRACT: The identification of drugs by the combination of two-dimensional HPTLC and remission spectrometry is described. The discriminating power and information content of this combination are calculated and compared with the corresponding values of GC/MS. Advantages and disadvantages of HPTLC and applications to some drugs are mentioned.

KEY WORDS: Two-dimensional HPTLC; Remission spectra; Discriminating power.

Problems of Forensic Sciences, vol. XLII, 2000, 64–74
Received 9 September 1999; accepted 16 May 2000

Since the fundamental works of Stahl [15] over 40 years ago thin layer chromatography has been firmly established in analytical chemistry and some years later in toxicological-chemical analytics too, though its importance may often be exceeded by GC/MS and HPLC.

Although its simple performance (detection of substances by rf-value and a lot of spray-reagents, e.g."Toxilab" (Ansys Diagnostics, Inc., USA) and "Spot-check"-system [14]) is one of the main reasons of its still existing attractiveness, there was no stagnation in further developing of TLC-technique. Today the use of high performance separation layers (HPTLC, especially with spheric particles ("Lichrospher" by Merck, Germany), the automated multiplied development (AMD-technique) and the use of automatic applicators and scanners (including remission spectrometry) led to better separation and lower limits of detection.

Especially the remission spectrometry permits an identification of drugs analogous to the HPLC-DAD-equipment, although the remission spectra show some disadvantages in comparison to spectra measured in solution [4]:

1. lower intensity at short wavelengths;
2. the spectral shape is less pronounced;
3. the spectral shape depends on the respective concentration (mass per TLC-spot);
4. no simultaneous registration of chromatogram and spectrum.

On the other hand the general characteristics of TLC (fast registration of several chromatograms on one plate and the easy change between quite different

chromatographic conditions (e.g. high polar – non polar or acid – basic) have a noticeable beneficial effect.

Whereas the lower intensity at shorter wavelengths and the poorer spectral shape can not be influenced, the dependence of spectral shape on concentration can be considered in the case of drug identification. We consider this dependence in such a way, that from each substance the remission spectrum is measured at four different concentrations (e.g. at 400, 800, 1200 and 1600 arbitrary units). These four spectra are stored in the database.

Attempts and experiences over many years have shown, that an identification of substances is not possible or possible only to a restricted extent, if the database contains only one remission spectrum per substance – in contrast to Ojanperä and co-workers [9, 10, 11].

For example the spectrum of sulpiride in an overdose case showed a FIT-value of 0.027 (ideal 0.000) in comparison to the most intensive spectrum in the database, but only a FIT of 0.199 in comparison to the lowest spectrum (rank 1 and 32, resp.).

On the other hand, verapamil at therapeutic drug monitoring shows FIT-values of 0.033 and 0.255 respectively in comparison to the lowest and the highest spectrum in the database. The latter FIT is comparable with codeine, which possesses a quite different spectrum.

The relatively small separation power of the combination TLC/UV (comparable with or even lower than HPLC-UV, Remedi-system) results in a restricted effectiveness.

The problem of overlapping can partly be solved by additional using of rf-values for drug identification [8, 12, 17] or measurement of spectra on back and front of the peak. The peak purity can be discovered by automatic chromatogram registration at three wavelengths (e.g. 210, 260 and 310 nm).

The separation power of TLC and HPTLC can be enhanced considerably by two-dimensional development, as can be shown by a simple assessment:

	HPTLC (1-dim)	HPTLC (2-dim)	Capillary-GC	HPLC (REMEDI)
Distance	100 mm/2.5 mm	100*50/2.5*2.5	900 s/3 s	13 min/0.2 min
Peak wide	= 40	= 800	= 300	= 65

This rough assessment shows that it is worthwhile to investigate the separation power, i.e. the discriminating power and information content, of two dimensional HPTLC for a distinct lot of toxicologically relevant substances and to compare it with capillary GC/MS (the DP of HPLC is much smaller and a comparison is not so worthwhile).

We investigated 50 basic drugs (psychoactive drugs, hypnotics, β-blocking agents a.o.), ethylacetate/methanol/ammonia (85/10/5) [16] and chloroform/methanol (90/10) [1] were used as mobile phases. Most of the rf-values for chloroform/methanol had to be determined experimentally, as well as the peak-half widths and some retention indices

of newer substances for GC liquide phase OV-1 [13]. Approximate values of the limits of detection were calculated from calibration curves or results of authentic cases. Table I shows exemplary the used data of five drugs.

TABLE I. USED DATA OF FIVE DRUGS

Drug	rf EMA	Rf CM	b_H HPTLC [mm]	LOD HPTLC [ng/spot]	rt GC/MS [s]	b_H GC/MS [s]	LOD GC/MS [ng/injection]	Therap. conc. [ng/ml]
Amitriptyline	69	19.5	2	15	1029	2	0.1	100
Citalopram	58	10.4	2	15	1128	2	0.1	30
Metoprolol	44	5.2	2	20	946	5	5	200
Midazolam	60	53	3	15	1212	8	2	50
Nitrazepam	64	53	3	15	1283	15	5	50

The discriminating power: DP = 1–2M/N(N–1) [16] was calculated according to different procedures (Table II):

1. by means of retention-window;
2. by peak-width (rf-value or retention time respestively constant);
3. by standard deviation F of the retention values [5, 8];
4. by peak width and standard deviation (strongest and most realistic criterion).

TABLE II. CALCULATED VALUES OF DISCRIMINATING POWER

Methods	rf-window: 5 rt-window:10	Peak half width b_H	Standard dev. σ-rf, δ-rt	b_H and σ-rf, σ-rt
HPTLC-EMA 1-dimensional	0.776	0.898	0.834	0.803
HPTLC-EMA+CM 2-dimensional	0.953	0.990	0.973	0.963
Capillary GC/MS	0.962	0.977	0.980	0.970

The fact that the retention times in the case of GC/MS have essentially smaller variations than the rf-values in the case of HPTLC was taken into consideration.

The preliminary results show clearly that the discriminating power of two-dimensional HPTLC is comparable with capillary gas chromatography not only in theory.

The comparison becomes even more realistic, if the method of detection (UV and MS, respectively) is considered during the calculation of peak overlapping. For example, if in case of GC/MS the substance under study has a base peak at 300 and the interfering substance has no mass peak at 300, then the interfering equals zero – despite overlapping! This is valid for remission spectra too, but in this case the lower specifity of UV-VIS-spectrometry reduce the discriminating power in comparison to GC/MS.

More meaningful than DP regarding the efficiency of an analytical procedure is the information content (eq. 1) [2], which besides the overlapping also considers the detectibility of the drugs.

$$I_i = \frac{(N - M_i)}{(N-1)} \cdot Id \left[\frac{\left(c_i - c_{i,\min} \right)}{\sigma_{ci} \sqrt{2\Pi e}} \right]$$

The information content of a quantitative analytical procedure of substance "i" is determined by the desired concentration c_i, the minimal detectable concentration $c_{i,\ min}$ (Table I) and the error σ_{ci} of the procedure. In the following considerations the coefficient of variation is fixed at 5%, i.e., $\sigma_{ci} = 0.05\ c_i$. C_{min} corresponds to the limit of detection or quantification (LOD, LOQ), the recovery and the aliquots, which are injected in GC/MS or applicated on HPTLC-plate are considered for the calculations. If the logarithmic expression in equ. 1 is smaller than zero then it is fixed at zero, all other values are normalized to 1.

The overlapping will be considered by means of a factor, where N is the number of drugs under study and M_i is the total amount of matches of a substance "i" with all other substances in the database. If $M_i = 1$ (overlapping only with itself), then the factor becomes 1, in the – not realistic – case of overlapping of all substances it becomes zero!

The ideal value of information content is equal to N (N = 50), which means that all substances under study are unequivocally detectable and quantitatively ascertainable without mutual interferences.

Table III shows the number S of drugs detectable by HPTLC and GC/MS (logarithmic expression > 0) as well as the information content (without and with consideration of overlapping).

TABLE III. INFORMATION CONTENT (IC) AND NUMBER OF DETECTABLE DRUGS

Methods	Number S	IC without overlapping	IC + overlapping retention window	IC + overlapping $b_H + \sigma$
HPTLC one-dimensional	40	31.40	24.28 (window = 5)	25.13
HPTLC two-dimensional	40	31.40	29.90 (window = 5)	30.17
Capillary GC/MS	42	37.68	36.35 (window = 10)	36.68

Table III again demonstrates the considerable gain of information resulting from the transition of one-dimensional to two-dimensional HPTLC because of substantially smaller overlapping, though the IC-values of two dimensional HPTLC are smaller than the values of GC/MS, because of the better detectibility (lower limits of detection) of numerous substances by means of GC/MS. The number S of drugs detectable by HPTLC is comparable with GC/MS.

Both methods complement one another advantageously, because drugs (Haloperidol, Temazepam) well detectable by HPTLC are poorer to record by GC/MS and vice versa (Tilidine, Fentanyl).

I want to appeal to the manufacturers of TLC-equipments to take care of the problem of automatic registration of two-dimensionally developed plates with optimization of individual TLC-spots and registration of their remission spectra at the same time. Then the HPTLC appears to be a true alternative in toxicological analysis and its indisputable advantages are entirely usable.

At present with the aid of the densitometer CD 60 (Desaga, Germany) we perform an automatic registration with following optimization and registration of spectra. This procedure is relatively time consuming (especially if a lot of spots are to registered), but has stood the test in different applications.

The following practical advantages and disadvantages of (two-dimensional) HPTLC – especially in comparison to HPLC (same detection: UV and fluorescence) – are to be mentioned [6]:

Advantages:

- Discriminating power is considerably higher.
- Fast change of chromatographic conditions is possible.
- No memory effects and no compulsion for elution.
- No alteration and contamination of columns and detectors calibration curves at each measurement.
- Simultaneous development of several chromatograms, i.e. shorter times of analyses.
- Postchromatographic derivatization is possible (e.g. gain of fluores- cence).
- One chromatogram can be registered by several detectors.
- No interference of detection by mobile phase.
- If matrix does not interfere – no expensive clean up is necessary.

Disadvantages:

- Despite autosamplers und applicators there is no full automatization (overnight) possible.
- Smaller intensity at shorter wavelengths and smaller marked spectral shape.
- Spectral shape depends on concentration.
- Often no linear calibration curve, i.e. smaller measuring range.
- More sensitive to overloading.
- Two dimensional distribution in the layer of separation is possible.
- Simultaneously registration of chromatograms and spectra still impossible at the present.
- Smaller reproducibility of retention.
- Measurement including internal standard is uncommon.

It is clear, that an analytical procedure with such high values of discriminating power and information content is particularly suited for general unknown cases, e.g. for investigation of autopsy cases without directed suspicion. Because of the high separation

power there is also a poor interference by biological matrix. A great advantage in the case of investigation of putrified material is, that there no contamination of columns or detectors can occur and immediately after the analysis of such material an analysis can be performed, which requires highest purity and sensitivity, e.g. the determination of haloperidol in serum.

Because of high separation power an interference by comedication (clozapine, levomepromazine and their metabolites in the investigated case) can be ruled out, on the other hand its detection is also possible. Figure 1a demonstrates the determination of haloperidol concentration and Figure 1b its unequivocal detection.

The determination of antiarrhythmics and β-adrenergic antagonists can be carried out as well. Because of their high polarity a proteine precipitation was used for their isolation, matrix interferences [7] because of the proteine precipitation can be fully avoided by means of two dimensional development. Also, for detection and quantitation of benzodiazepines HPTLC is quite useful, for these relevant compounds the above-mentioned completion with GC/MS (without derivatization) proves to be of particular advantage. The intensive fluorescence of some compounds (e.g. zolpidem, zopiclone and olanzapine) permits a very sensitive detection of these drugs, as demonstrated at the previous symposium of the GTFCh 1999 in Mosbach [3].

More than 60 drugs, the determination of which was demanded from our laboratory and performed with the aid of TLC and HPTLC, are shown in Table IV.

TABLE IV. DETERMINATED DRUGS USING TLC AND HPTLC

Alizapride	Diazepam	Metronidazol	Risperidone
Alprazolam	Diclofenac	Mirtazapine	Sotalol
Amiodarone	Doxepine	Nefazodone	Sulfamethoxazol
Amisulpride	Droperidol	Moclobemide	Sulpiride
Amlodipine	Flecainide	Nitrazepam	Talinolol
Atenolol	Flucytosine	Nordazepam	Temazepam
Bromazepam	Flunitrazepam	Olanzapine	Tetrazepam
Carbamazepine	Fluoxetine	Opipramol	Tiapride
Carb.-Epoxide	Fluvoxamine	Oxcarbazepine	Triamterene
Celiprolol	Furosemide	Paracetamol	Trimethoprim
Quinidine	Haloperidol	Paroxetine	Venlafaxine
Chloramphenicol	Ibuprofene	Phenazone	Verapamil
Chlordiazepoxide	Indometacine	Phenprocoumon	Zolpidem
Clobazam	Lamotrigine	Pipamperone	Zopiclone
Clonazepam	Maprotiline	Propafenone	
Clozapine	Metoclopramide	Pyrazinamide	
Caffein	Metoprolol	Ranitidine	

TLC is especially appropriate for the two most required therapeutic drug monitoring procedures in our laboratory: clozapine (Figure 2a) and lamotrigine (Figure 3a). For

these two very conveniently detectable substances (λ = 290 and 320 nm, respectively) a simple TLC is sufficient. The registration of chromatograms of several cases (Figure 2a) requires only some minutes including the registration of spectra to confirm, that measured peaks are really clozapine or lamotrigine (Figure 2b and 3b, respectively). The ap-

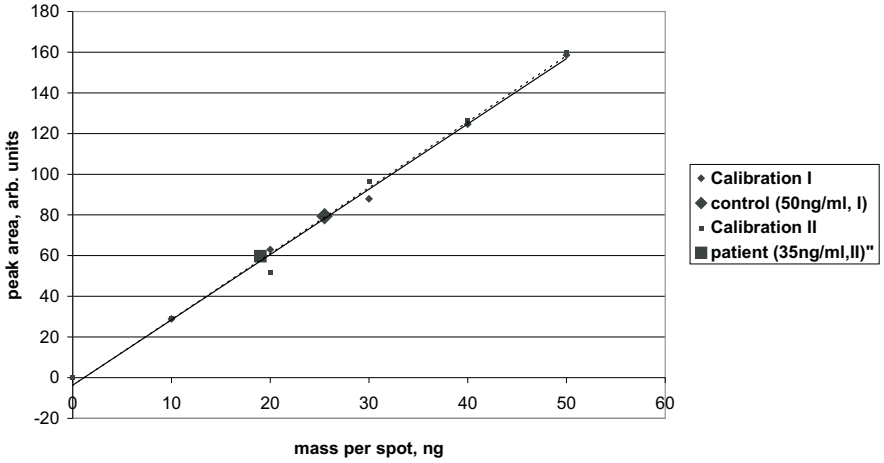

Fig. 1a. Determination of Haloperidol.

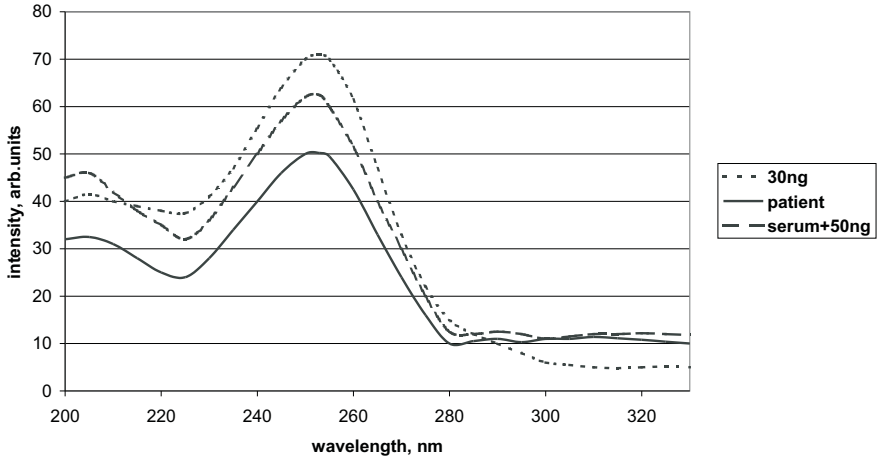

Fig. 1b. Detection of Haloperidol.

plication was performed automatically as in the case of all TLC-analyses by means of the applicator TLS-100 (Baron, Germany).

Summarizing it can be said, that HPTLC because of its high discriminating power and information content, its great variety and its technical developments (especially, if the mentioned combination between two dimensional registration and multiple diode array detection would be realized) is by no means "a chromatography of the underdog".

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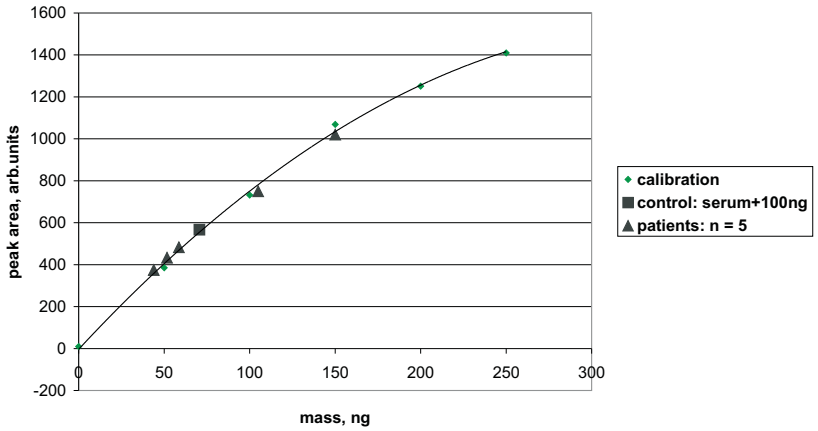

Fig. 2a. Determination of Clozapine.

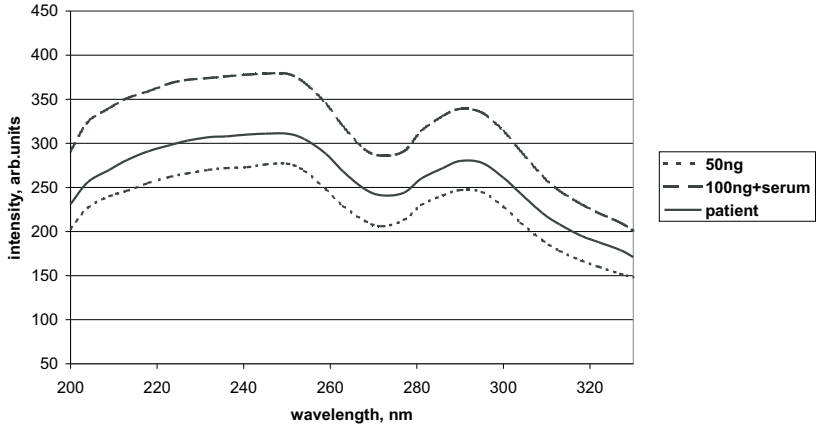

Fig. 2b. Detection of Clozapine.

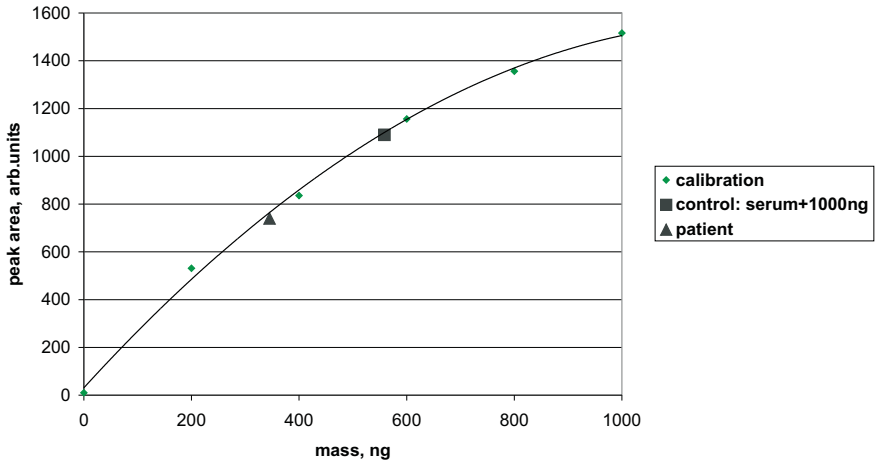

Fig. 3a. Determination of Lamotrigine.

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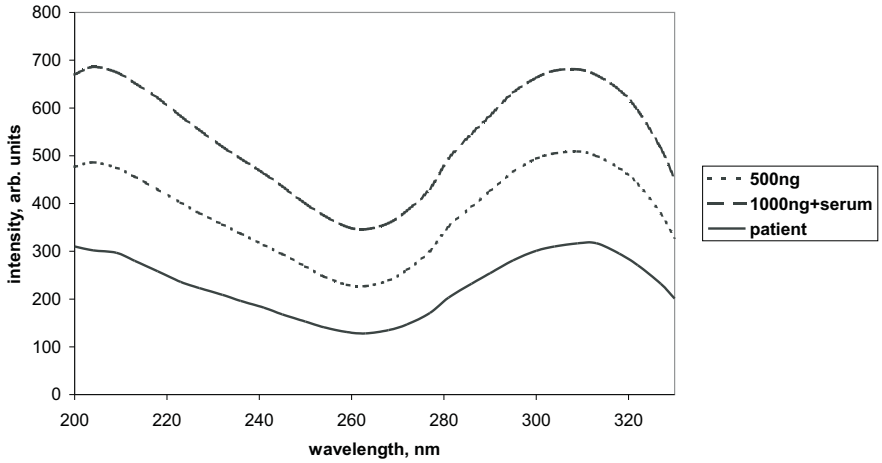

Fig. 3b. Detection of Lamotrigine.

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