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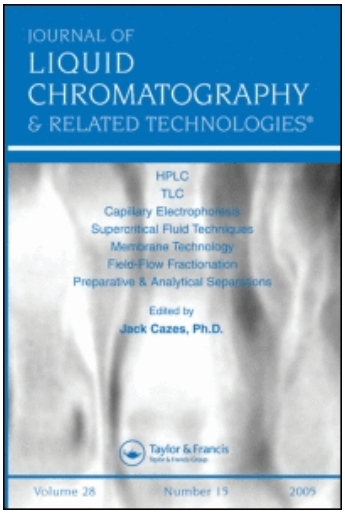

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Experience with Quantitative Thin-Layer Chromatography in the Analysis of Drugs in Plasma

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EXPERIENCE WITH QUANTITATIVE THIN-LAYER CHROMATOGRAPHY IN THE ANALYSIS OF DRUGS IN PLASMA

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ABSTRACT

A survey is presented concerning the possible techniques of quantitative thin-layer chromatography. Most of the possibilities mentioned have been used successfully in our laboratory for the determination of drugs and metabolites in plasma. With respect to our own experience , the advantages and disadvantages of quantitative thin-layer chromatography are discussed.

Although gas chromatography and high-performance liquid chromatography are more popular, quantitative thin-layer chromatography has a high potential in the analysis of drugs in samples of biological origin and should not be neglected in the development of an assay.

INTRODUCTION

Pharmacokinetics is the study of the time course of absorption, distribution, metabolism and excretion of a drug in the organism. To obtain this information it is necessary to determine the levels of the unchanged drug and its metabolites in different fluids,

tissues and excreta of the body. The highly active pharmacological substances of today, which require doses of several milligrams or even less to be effective, often yield very low concentrations in the biological materials.

The most sensitive methods have to be used to determine the levels in the nanogram range. In recent years, we have successfully used the in situ quantitation of thin-layer chromatograms for this purpose. We have developed many assays for drugs in blood or plasma samples. Some of these methods have been published and show the broad application range of this technique.

In this paper we compile our experience with quantitative thin-layer chromatography and compare the advantages and disadvantages of this method.

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GENERAL REMARKS

In the beginning, quantitation of substances after thin-layer chromatography was performed by scraping off the adsorbent, elution and spectrophotomectric quantitation of the substance (1,2,3). These procedures are today of a secondary value, since they are time-consuming and require large amounts of the substance to be determined. In pharmacokinetic studies these quantities are not available in most cases. For more than 10 years in situ quantitation has been possible by direct scanning the plate with commercial instruments. But contrary to high-performance liquid chromatography, this technique has not reached the same evolution and distribution. Perhaps this is a result of the problem of correlating the scanner reading to the amount of substance on the plate.In addition, the early instruments were

rather expensive. But with the set-ups of today, which have a high sensitivity, it is possible to apply low concentrations avoiding overloading of the thin-layer. Simple linear correlations exist in this range between the amount on the thin-layer and the signal measured with the scanner (4). Moreover, the recently available thin-layer scanners are low-price instruments with good characteristics, therefore this technique should regain more popularity.

TECHNIQUES USED FOR IN SITU QUANTITATIVE THIN-LAYER CHROMATOGRAPHY IN DETERMINATIONS OF PLASMA LEVELS

We used several techniques for the determination of plasma levels of drugs with thin-layer chromatography.

We have obtained the best densitometric results with the commercial precoated thin-layer plates, since the adsorbent layers are more homogenous than those of home-made origin. It is important for the manufacturer to guarantee the constant quality of his products. Each change in the production mode or the material used should be announced clearly. The user of precoated plates for quantitative thin-layer chromatography expects an extensive quality control performed by the manufacturer.

In most cases we have scanned the chromatograms with a Zeiss chromatogram spectrophotometer, model PMQ II.

Direct Reflectance Measurement

The direct reflectance measurement of UV-absorbing or colored substances on thin-layer chromatograms is the most convenient scanning technique. According to our experience , substances with an $E^{1\%}_{1\ cm}$ value

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of 800-1000 are detectable in amounts of 5 ng or less per spot, if the spot-diameter is kept small (3-5 mm). This requirement can often be fulfilled utilizing a proper application solvent.

In general, we have found a linear correlation between the amount on the thin-layer and the peak height of the scanner signal in a range of about 20 times the detection limit of a substance (5).

The direct reflectance measurement of thin-layer chromatograms for pharmacokinetic purposes is only applicable if endogenous interferences can be avoided either by an effective clean-up procedure or by choosing a highly selective solvent system. In the case of bromazepam, we obtained good results with this method down to 5 ng/ml plasma (5).

In addition, more complicated problems can be solved, but with more extensive sample preparations, as illustrated in the measurement of plasma levels of tricyclic drugs and their metabolites, with detection limits of 5-10 ng/ml in plasma (6).

The reproducibility of quantitative thin-layer chromatography is sufficient for pharmacokinetic studies. With bromazepam we have calculated the relative standard deviation of the entire assay, analysing the same unknown plasma sample two or more times on different days. We found a value of $\pm$ 6% down to 5 ng/ml. This value includes all parameters, such as variation of the extraction yield and variation of the calibration curve. Comparable reproducibilities were obtained with gas chromatography and high-performance liquid chromatography.

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Direct Fluorescence Measurements

In the analysis of plasma samples with quantitative thin-layer chromatography, substances with an intrinsic fluorescence should be measured fluorometrically, even if they have a high UV absorbance. The fluorescence of a substance is more specific than the UV absorbance, since its excitation and emission wavelength can be used for increasing the selectivity. Interferences by endogenous plasma components can be reduced . The clean-up of plasma extracts is less critical than in the direct reflectance measurement, and can be rather simple (7).

The intrinsic fluorescence of several substances is very intense and allows the detection of low amounts on thin-layer plates. The coumarin derivative phenprocoumon gave distinct fluorescence signals down to 0.1 ng per spot (7). It was possible to develop a highly specific and sensitive assay for this substance, and a reproducibility for the procedure was obtained of ± 3% down to 20 ng/ml.

An interesting improvement for the direct fluorescence measurement of thin-layer plates has been published by Steyn (8). Dipping the chromatogram, after drying, in a solution of paraffin wax increased the fluorescence of the substances analysed by a factor of 8, moreover this treatment had a stabilizing effect on the fluorescence. Preliminary experiments in our laboratory showed similar results.

Quantitation after Derivatization on Thin-Layer Plates

There exist two possibilities for using derivatization in thin-layer chromatography. Either the sub-

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stances are modified before the chromatographic step, or the reaction is performed post separation. We prefer the post-separation technique, since derivatization by-products do not interfere with chromatographic separation

Through simple chemical reactions, drugs and metabolites which show a low UV absorption and no intrinsic fluorescence, can be modified into products easily measured on thin-layer plates. If these reactions are specific, often endogenous components of the plasma extracts show no interferences in densitometric evaluation of the chromatograms.

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Post-separation derivatization in qualitative thin-layer chromatography is well-known. Numerous spray reagents for the different chemical substance-classes are commercially available. But not all of these derivatization reactions are suitable for quantitative evaluation. The derivatives formed on the thin-layer must be completely stable until the chromatogram will have been scanned. This requirement is only fulfilled by a limited number of spray reagents.

We have performed extensive studies with the well-known color reaction of Bratton and Marshall (9), which is specific for primary aromatic amines. The reaction yields azo-dyes with a high extinction coefficient, allowing the detection of low quantities of primary aromatic amines. Furthermore, these dyestuffs are sufficiently stable on silicagel thin-layers for densitometric scanning. The Bratton-Marshall reaction has been widely used for the determination of sulfonamides in biological materials. But there are also benzodiazepines having no substituent in the N-1 position, which can be hydrolysed to primary aromatic amines, forming azo-dyes. The

thin-layer chromatographic assay of bromazepam was rendered more specific by this procedure (5).

The amino metabolites of the 7-nitro-benzodiazepines undergo the Bratton-Marshall reaction as well. A method has been established for the determination of these metabolites in plasma (10).

We have developed a rather extensive derivatization procedure for the determination of chlorpheniramine (11) and amitriptyline (12) in plasma. The compounds were first nitrated on the thin-layer plate, then the reduction of the nitro derivatives led to aromatic primary amines, which gave the Bratton-Marshall reaction. In both methods, four consecutive sprayings of the thin-layer plates were necessary; reproducibility: ± 8 %.

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Fluorescent derivatives with a good stability on silicagel thin-layers are produced with the reagent fluorescamine. For the detection of flunitrazepam and its major metabolites in plasma, an assay using fluorescamine was established (13). A high sensitivity (about 1 ng/ml and less) could be obtained, but as this reagent reacts with aromatic and aliphatic primary amino-groups, we had severe problems with respect to endogenous plasma components. If primary aromatic amines had to be determined, we found less interferences with the Bratton-Marshall reaction.

The reagents can be applied to the thin-layer either by dipping the plate or by spraying it. To date, we have had no success when dipping the thin-layer in the reagent solution. To spray a thin-layer plate homogeneously requires considerable experience of the technician. Recently a commercial spraying device has been made available (14), which gives improved uniformity in comparison to manual operation. Never-

theless, we have obtained good reproducibilities even with manual spraying (11, 12).

Some of the derivatization reactions have to be performed at elevated temperature. It is of great importance to attain throughout the whole adsorbent layer a uniform and defined temperature. For this purpose we have used a drying oven equipped with a large metal block with a high heat capacity.

We are sure that for the determination of drugs and metabolites in plasma, the post-separation derivatization technique has a wide range of application.

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ADVANTAGES AND DISADVANTAGES OF QUANTITATIVE THIN-LAYER CHROMATOGRAPHY IN THE ASSAY OF PLASMA SAMPLES

Advantages

In thin-layer chromatography each run is performed on fresh adsorbent. This is an important advantage in the analysis of plasma samples, where a drug or its metabolites have to be determined in presence of a large excess of endogenous components. In gas chromatography and liquid chromatography the same columns are used for a large number of analyses. Even with efficient clean-up procedures the plasma extracts injected onto the column contain large amounts of plasma constituents loading the adsorbent. They remain to a certain extent on the column or elute very slowly, leading to interfering peaks. In addition, the separation property of a column is influenced by these endogenous substances.

A thin-layer chromatogram gives a complete view of the entire plasma extract applied. A similar survey is impossible with gas or liquid chromatography.

We have found that quantitative thin-layer chromatography is less sensitive to impurities of the extraction and separation solvents than liquid chromatography. The reason is that in thin-layer chromatography the chromatogram is dried after the development and all volatile impurities are evaporated. Furthermore, in liquid chromatography with UV detection, solvents with a high UV absorbance cannot be used, contrary to thin-layer chromatography, where the mobile phase is removed from the adsorbent after separation by drying.

In one run, on thin-layers a series of samples is separated, whereas in the other chromatographic techniques one sample after another has to be separated.

On a thin-layer chromatogram only the interesting zone has to be scanned, it is not necessary to measure the whole length of run. Therefore, with the new devices the time for one analysis is relatively short.

Derivatization on thin-layers is possible with aggressive reagents such as concentrated mineral acids, and no additional apparatus is necessary in contrast to liquid chromatography.

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Disadvantages

The migration distance of 10-15 cm on a thin-layer allows only the separation of a limited number of substances. In the analysis of plasma extracts with numerous interfering components, this is a serious drawback.

The range of a linear correlation between the amount of a substance on the layer and the signal measured, is small compared to liquid chromatography.

Full automation of thin-layer chromatography is very difficult. To date, only instruments for the individual steps, such as application of the samples, densitometric measurement of the chromatograms, have proven to be reliable.

The most severe disadvantage of quantitative thin-layer chromatography is the instability of several substances on silicagel, the most widely used adsorbent. Even some quite stable substances are degradated in contact with silicagel. Precoated thin-layer plates with inert application zones are commercially available. But these systems are not applicable in each case. On the inert application zones it is difficult to get small spots, which is of importance to obtain a high sensitivity. In one case we succeeded with a special treatment of the adsorbent (12). Perhaps the new contact spotting device (15) will help to overcome this stability problem. In any case this device seems to be an improvement in quantitative thin-layer chromatography dealing with samples of biological origin.

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CONCLUSIONS

Quantitative thin-layer chromatography is a recommendable analytical method for pharmacokinetic studies. Due to the rapid growth of high-performance liquid chromatography, densitometry has not reached the appreciation it merits; but the new possibilities such as high-performance thin-layer plates and the low-price scanners may help to intensify research in this field.

The simplicity and the specifity of the derivatization techniques are important aspects of quantitative thin-layer chromatography which, even devoid of being fully automated, is a rapid analytical tool.

After administration to humans, plasma levels of drugs and their metabolites are sometimes in the nanogram range. To develop sensitive assays, extensive experiments are necessary. We believe that it is worthwhile to consider not only gas chromatography and high-performance liquid chromatography, but also quantitative thin-layer chromatography for this purpose. A comparison of the three techniques is important and allows the investigator to choose the most useful chromatographic method.

REFERENCES

1. Gänshirt, H., Untersuchung zur quantitativen Auswertung der Dünnschichtchromatographie, Arch. Pharmaz., 296, 129, 1963.

2. Haefelfinger, P., Schmidli, B., Ritter, H., Quantitative Bestimmung von Phenacetin, Isopropylantipyrin, Coffein und Persedon® in einem pharmazeutischen Kombinationspräparat, Arch. Pharmaz., 297, 641, 1964.

3. Haefelfinger, P., Quantitative Bestimmung eines quaternären Alkaloides nach der dünnschichtchromatographischen Trennung, J. Chrom., 33, 370, 1968.

4. Touchstone, J.C., Schwartz, M.F., Levin, S.S., Quantitation of Phenobarbital and Para-Hydroxy-Phenobarbital: A Study in Densitometry of Thin-Layers, J. Chrom. Sci., 15, 528, 1977.

5. Haefelfinger, P., Determination of Bromazepam in Plasma by Quantitative Thin-Layer Chromatography, Chromatographia, 11, 10, 1978.

6. Breyer, U., Villumsen, K., Measurement of Plasma Levels of Tricyclic Psychoactive Drugs and Their Metabolites by UV Reflectance Photometry on Thin-Layer Chromatograms, Europ. J. Clin. Pharmacol., 9, 457, 1976.

7. Haefelfinger, P., A Specific and Sensitive Method for the Determination of the Anticoagulant Phenprocoumon in Plasma, J. Chrom., 162, 215, 1979.

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8. Steyn, J.M., A Thin-Layer Chromatographic Method for the Determination of Acebutolol and its Major Metabolite in Serum, J. Chrom., 120, 465, 1976.

9. Bratton, A.C., Marshall, E.K., A New Coupling Component for Sulfonamide Determination, J. Biol. Chem., 128, 537, 1939.

10. Haefelfinger, P., Determination of the 7-Amino-Metabolites of the 7-Nitro-Benzodiazepines in Human Plasma by Thin-Layer Chromatography, HRC & CC, 2, 39, 1979.

11. Haefelfinger, P., Determination of Nanogram Amounts of Aromatic Compounds by Spectrophotometry on Thin-Layer Chromatograms, J. Chrom., 124, 351, 1976.

12. Haefelfinger, P., Determination of Amitriptyline and Nortriptyline in Human Plasma by Quantitative Thin-Layer Chromatography, J. Chrom., 145, 445, 1978.

13. Haefelfinger, P., Determination of Nanogram Amounts of Primary Aromatic Amines and Nitro Compounds in Blood and Plasma, J. Chrom., 111, 323, 1975.

14. Kreuzig, F., Gerät zum gleichmässigen Besprühen von Dünnschichtplatten mit Reagentien für die quantitative Dünnschicht-Chromatographie, Z. Anal. Chem., 282, 457, 1976.

15. Fenimore, D.C., Meyer, C.J., A New Approach to Sample Application in Thin-Layer Chromatography, in: Advances in Chromatography, Proceedings of the 14th Int. Symp., Lausanne, Elsevier, Amsterdam, 1979, p. 583.

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