Determination of Drugs in Plasma by High-Performance Thin-Layer Chromatography

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High-performance thin-layer chromatography was used to determine chlorpromazine, amitriptyline, nortriptyline, imipramine, desipramine, phenobarbital, and phenytoin in plasma, to demonstrate the utility of this technique for routine analysis. We quantitated the separated components by use of ultraviolet reflectance spectrometry with detection limits as low as 1 μg/liter. Regressions of psychoactive agents extracted from plasma were linear over the range of 0 to 300 μg/liter. The anti-convulsant drugs, phenobarbital and phenytoin, were determined over a range of 0 to 50 mg/liter. Analyses were rapid, reproducible, and well-suited to large-scale programs. Separated components also can be identified in situ by ultraviolet spectrophotometry.

Additional Keyphrases: drug assay • monitoring therapy • toxicology

As evidence accumulates correlating concentrations of psychopharmacologic agents in the blood with treatment efficacy, there is increasing interest in analytical methods which could be applied to large clinical populations. Most methods currently available, however, require techniques and instrumentation that are at a level of sophistication not always compatible with routine, large-scale programs. A high order of sensitivity is usually necessary, because many of these compounds are present therapeutically in concentrations in the low μg/liter range, and the situation may be further complicated by the presence of metabolites that are similar in structure to the parent compound and are a potential source of interference.

For these reasons, chromatographic separations used with sensitive detection systems have been predominant in assays of drugs in blood and other biological samples. Such techniques as electron-capture gas chromatography (1–4), nitrogen-sensitive gas chromatography (5, 6), mass fragmentography (7, 8) and, more recently, high-performance liquid chromatography (HPLC) with electrochemical detection (9) all can provide the requisite sensitivity and selectivity.

The major disadvantage of these methods, especially for routine clinical determinations, is the considerable instrument time involved in sequential separations necessitated by column-chromatographic systems. Thin-layer chromatography (TLC), on the other hand, permits simultaneous separation of many samples. Drugs in blood have been so estimated, with densitometry for several years, but the problem of attaining still-greater sensitivity has restricted this technique to drugs that are present in fairly high concentrations, such as the anticonvulsants (10), or to use of large sample volumes if the drug is at low concentrations, as is the case with chlorpromazine (11).

Recently introduced materials and methods for TLC have improved both resolution and sensitivity of detection to the point that the appellation “high-performance” may justifiably be used. Rippahn and Halpaap (12, 13) described high-performance thin-layer chromatography (HPTLC) and the characteristics of HPTLC plates coated with silica gel of carefully controlled particle size distribution, which yielded substantially better separations than other thin-layer plates. Kaiser discussed means by which samples could be applied to the plate in HPTLC (14), and Hezel reported on quantitative measurements performed with HPTLC (15). This method was used by Ritter in the determination of the diuretic, muzolamine, in plasma and urine (16), and Fenimore et al. described the application of HPTLC to antipsychotic drugs (17).

This present report, a continuation of the latter study, deals with the measurement of chlorpromazine and its metabolites and of tricyclic antidepressants in blood. Studies of the anticonvulsant drugs phenobarbital and
phenytoin are included, for although these drugs are not strictly psychopharmacologic agents, they are frequently administered to patients receiving treatment at mental-health facilities.

Materials and Methods

Reagents


Solvents, from Fisher Scientific Co., were all distilled before use in all-glass systems and stored in bottles with poly(tetrafluoroethylene)-sleeved glass stoppers.

All glassware coming into contact with samples or extracts was silylated by use of hexamethyldisilazane at elevated temperature and reduced pressure (18).

Stock Solutions

Solutions containing internal standard (IS) and carrier (C) compounds were prepared for the following assays:

Chlorpromazine: per liter, 40 mg of butaperazine (IS) and 200 mg of perphenazine (C) in isooamyl alcohol/heptane (15/85 by vol).

Amitriptyline, nortriptyline, imipramine, and desipramine: per liter, 10 mg of loxapine (IS) and 200 mg of perphenazine (C) in isooamyl alcohol/heptane (15/85 by vol).

Phenobarbital and phenytoin: per liter, 100 mg of metharbital (IS) in ethyl acetate.

Extraction and sample preparation solutions include NaOH (1 mol/liter), HCl (50 mmol/liter), NH4OH (1 mol/liter), citrate buffer (1 mol/liter, pH 5), and isooamyl alcohol/heptane (1.5/98.5 by vol).

Development solvents were hexane/acetone/diethylamine, 77/20/3 by vol., and ethyl acetate/ammonium hydroxide 97/3 by vol.

Modified Forrest reagent, for making visible the phenothiazine drugs, was prepared according to Chan and Gershon (11) by dissolving 1 g of ferric chloride hexahydrate in 100 ml of sulfuric acid/water (1/1 by vol) and 300 ml of absolute ethanol.

Thin-Layer Chromatographic Materials

HPTLC plates (HPTLC silica gel 60) were from E. Merck, Darmstadt, G.F.R. Twin-trough development tanks, variable volume, and 100-ml platinum–iridium spotting pipettes were from Camag, Inc., New Berlin, Wis. Glass spotting pipettes were made by drawing disposable Pasteur pipettes with a flame and then silylating as above.

Spectrophotometry

A Model KM-3 Chromatogram Spectrophotometer (Zeiss Instruments, New York, N. Y.) was used for all quantitative measurements. Ultraviolet absorbance in the reflectance mode was measured for all compounds, with use of a 0.5-mm slit-width and 3.5-mm slit-length. Measurement wavelengths were as follows: chlorpromazine, 254 nm; amitriptyline and nortriptyline, 275 nm; and phenobarbital and phenytoin, 230 nm. Visible absorbance in the reflectance mode from chlorpromazine, after reaction with modified Forrest reagent, was measured at 425 nm.

Procedures

Sample Preparation

Chlorpromazine, amitriptyline, nortriptyline, imipramine, or desipramine. Deliver a 100-μl portion of stock solution (see above), diluted 10-fold and containing internal standard and carrier, to a 15-ml silylated screw-top test tube and evaporate under reduced pressure. Add 1 ml of plasma, 1 ml of NaOH (1 mol/liter), and 10 ml of isooamyl alcohol/heptane (1.5/98.5). Mix gently for 30 min with a tube- rocker and then centrifuge for 5 min in a bench-top centrifuge to separate the phases. Freeze the aqueous plasma layer in an acetone–solid CO2 bath, and transfer the organic layer to another 15-ml tube. Add 1 ml of the dilute HCl, mix with a vortex mixer, and separate by centrifugation. Aspirate the organic layer, and alkalinate the remaining aqueous layer to pH 10 by adding 0.2 ml of the dilute ammonium hydroxide. Extract with 2 ml of isooamyl alcohol/heptane (1.5/98.5) by mixing, then centrifuge. Freeze in the acetone–solid CO2 bath and transfer the organic phase to a silylated Reactivial (Pierce Chemical Co., Rockford, Ill.). Evaporate it under reduced pressure and re-dissolve the residue in 10 μl of isooamyl alcohol/heptane (15/85) immediately before spotting the HPTLC plate.

Phenobarbital and phenytoin. Add 400 μl of stock solution containing 100 mg of metharbital per liter to a 15-ml screw-top test tube and evaporate under reduced pressure. Add 1 ml of plasma, 1 ml of pH 5 citrate buffer, and 5 ml of diethyl ether. Mix gently on a rocker-type shaker for 30 min. Remove the ether phase to a Reactivial and evaporate under dry nitrogen at 40 °C. Dissolve the residue in 50 μl of dichloromethane and spot on an HPTLC plate.

Thin-Layer Chromatography

Psychopharmacologic agents. Spot the entire re-dissolved sample on the HPTLC plate at 1.0 cm from the edge, using a variable-volume platinum–iridium
pipette and taking care to keep the spot diameter to less than 2 mm. After spotting, place the plate under reduced pressure to assure complete removal of the spotting solvent. Place the plate in a twin-trough chamber containing the hexane/acetone/diethylamine solvent mixture for 5 min for equilibration and then tilt the chamber to allow the solvent to reach the plate. Allow the solvent to ascend 4 cm above the origin, and then place the plate under reduced pressure for 30 min to remove the solvent completely before scanning with the chromatographic spectrophotometer.

Anticonvulsant drugs. Spot sample on the HPTLC plate with the 100-nl platinum-iridium pipette. Develop as above, using the ethyl acetate/ammonium hydroxide mixture and allowing the solvent to run 4 cm above the origin. Dry thoroughly in warm air or reduced pressure before scanning.

Results

HPTLC, like any high-resolution separation technique, requires a certain amount of attention to detail for best results. Pre-washing the plates, application of the sample to as small an area as possible, and development in carefully controlled, pre-saturated chambers are all requisites to satisfactory and reproducible analyses. Nevertheless, with a minimum of experience separations can be attained such as that shown in Figure 1, with development times often measured in seconds rather than minutes. An appropriate TLC plate is, however, a necessary requirement, as seen in Figure 2. The resolution attained in these two separations is similar, but the background noise arising from irregularities in the surface of the conventional plate limits the sensitivity of measurement.

Figure 3 shows a scan of the HPTLC separation of chlorpromazine from a 1.0-ml plasma sample containing 10 μg/liter. The rising baseline is due to impurities absorbed by the silica gel that were not totally removed by pre-washing the plate; the material present at 4 cm from the origin is impurity that moved with the solvent front. This is frequently observed at high sensitivity but seldom causes interference. The linear regression of peak-height ratios of chlorpromazine to the internal standard, butaperazine, with concentration in blood plasma is illustrated by Figure 4. Therapeutic concen-
trations of the drug are well within the usable range of concentrations (2). With ultraviolet absorption as the means of quantitation, the limit of detectability of chlorpromazine in blood plasma is about 1 µg/liter, approximately the sensitivity achieved by electron-capture gas chromatography (4).

Several chromogenic reagents have been proposed for making phenothiazines visible on thin-layer plates, including Dragendorff spray, Marquis reagent, and FPN reagent (19, 20). Gershon et al. (11) used a modified Forrest reagent in studies of chlorpromazine and its metabolites, and this spray appeared to yield an intense, stable color that could possibly serve as an alternative to ultraviolet absorptionmetry, to enhance the sensitivity of detection of chlorpromazine. When examined quantitatively, however, the sensitivity was slightly less than that attained by ultraviolet absorption, and the intensity was observed to vary fairly quickly. Figure 5 illustrates the change in peak-height ratios of chlorpromazine to the internal standard some 30 min after color development; the increase in ratio is due primarily to the decrease in intensity of the internal standard with time, but both compounds faded almost completely in 1 to 2 h. Ultraviolet absorption, on the other hand, is relatively stable, but scans should nevertheless be completed as soon as possible after chro-

matographic separation, to avoid errors that might accompany degradation of the compound by light, atmospheric oxidation, or contamination.

The metabolic products of drugs are usually more difficult to determine than the parent compounds because of their increased polarity resulting from (e.g.) addition of hydroxyl groups or loss of methyl groups. Consequently, derivitization is usually a necessary step in gas chromatography, to prevent sample loss through decomposition or adsorption to active sites in the chromatographic system. This problem is not as severe with liquid-chromatographic techniques, whether column or thin-layer, and Figure 6 shows a HPTLC chromatogram of the major metabolic products of chlorpromazine in a single chromatographic separation. The components of the mixture are resolved almost to baseline throughout, and the solvent front is only about 4 cm from the origin.

The tricyclic antidepressants are very similar to the phenothiazines with respect to sample preparation and HPTLC procedures. The principle difference is in the use of loxapine rather than butaperazine as the internal standard. Figure 7 shows the ultraviolet scan of the developed amitriptyline and nortriptyline HPTLC separation. Inasmuch as nortriptyline is an active metabolite of amitriptyline, simultaneous determination of these drugs is an important consideration for clinical studies. The regression lines for amitriptyline and nortriptyline are shown in Figure 8. Linearity and standard error appear to be acceptable throughout the reported therapeutic range of plasma concentrations for these drugs (21, 22).

Addition of a carrier compound that structurally is similar to the compounds being measured significantly
improves both recovery and reproducibility, particularly at low concentration (4, 17). Perphenazine was chosen for phenothiazine and tricyclic assays, because it conveniently remains at the origin in the development systems described in this report. The effect of this carrier on the recovery and reproducibility of measurement is shown in Table 1. As would be expected, the influence of carrier is more pronounced at low concentration, but even at 100 μg/liter, the improvement is sufficient to recommend its use.

Determinations of antiepileptic drugs in blood have received a great deal of attention in recent years, and methods ranging from immunoassays to nearly every type of chromatography have been proposed and evaluated for clinical use (10). The therapeutic concentrations of most of these drugs in blood are sufficient that sensitivity of detection is not a major problem, but on the other hand, the capability of utilizing small sample volumes is often a distinct advantage. Figure 9 shows a scan of phenobarbital and phenytoin extracted from blood plasma, each being present at a concentration of 10 mg/liter. The actual amount of drug on the HPTLC plate was 40 ng of each, and the amount of metharbital, the internal standard, was 120 ng. The regressions for these compounds were somewhat curved, but predictable. Replicate determinations of 20 mg/liter samples of these drugs in plasma were undertaken to ascertain what operations in the procedure were the major contributors to error. The results of these determinations (Table 2) reveal that the spectrophotometry, as shown in the third column of figures, is not a very significant source of variability. Instead, the spotting step and the sample preparation appear to be the main contributors to error. These procedures, however, are probably the most amenable to improvement as the operator becomes more expert.

![Fig. 7. Chromatogram of amitriptyline and its demethylated metabolite, nortriptyline, with loxapine as the internal standard (I.S.) Development solvent: hexane/acetone/diethylamine (77/20/3). Scanned at 240 nm.](image)

![Fig. 8. Linear regressions of amitriptyline and nortriptyline to loxapine (I.S) peak-height ratios at different plasma concentrations](image)

**Table 1. Recovery and Reproducibility of HPTLC**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc. in serum μg/liter</th>
<th>Recovery, %</th>
<th>Reproducibility (CV)</th>
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<tr>
<td></td>
<td>With carrier</td>
<td>Without</td>
<td>With carrier</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td></td>
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<td>96</td>
<td>87</td>
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<td>10</td>
<td>80</td>
<td>54</td>
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<tr>
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<td>99</td>
<td>87</td>
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<tr>
<td>10</td>
<td>95</td>
<td>71</td>
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<tr>
<td>Nortriptyline</td>
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<td></td>
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<td>100</td>
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<td>73</td>
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<td>10</td>
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<td>86</td>
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</table>

* Average values from six determinations.
Discussion

Although good resolution, sensitivity, and reproducibility are all achieved by HPTLC these requisites for determining drugs in blood can be met by most chromatographic techniques if sufficient attention is given to the components and operation of the systems.

Nevertheless, certain features of HPTLC merit attention other than the obvious advantage of simultaneous separations. To begin with, the selection of solvent systems for HPTLC is unrestricted by considerations of transparency to ultraviolet radiation, a major factor in choosing solvents for use with HPLC where ultraviolet absorption is the basis of detection. In addition, the compound being measured need migrate only enough to be separated from adjacent compounds. In contrast, with column chromatography the compound must traverse the length of the column, and all other components of the sample mixture must be eluted to avoid interference with subsequent samples. Also, sample clean-up does not have to be as meticulous with HPTLC, because there is no problem with column degradation due to the accumulation of components at the inlet.

Perhaps the most important feature of thin-layer systems is in the static rather than dynamic nature of the detection process. The components separated by TLC remain unmoving on the plate, to be examined by any of a number of methods at any time (stability being the limiting factor here). For example, an isolated spot can be characterized by scanning with reflectance ultraviolet spectrophotometry with incremental changes in wavelength. The ultraviolet spectra of chlorpromazine and chlorpromazine sulfoxide obtained in this manner are shown in Figure 10. We used such identification while studying multiple-development systems (i.e., re-running plates in the same solvent repeatedly) for chlorpromazine separation. An extraneous peak was observed at the position on the plate occupied by chlorpromazine before each re-development. This compound, indicated by the small arrow in Figure 11,
when subjected to in situ spectrophotometry, yielded the spectrum shown to the right of the chromatogram. Comparison to the spectrum of chlorpromazine sulfoxide in the previous figure reveals identical absorption maxima, and there is little doubt that chlorpromazine was oxidized on the plate under the conditions used in the multiple-development process.

Finally, with static detection it is also possible to examine portions of a chromatogram with such computer-assisted processes as signal averaging (23), digital filtering, edge detection, or pattern recognition—techniques not available for, or quite difficult to adapt to, detectors monitoring flowing column effluents.

References


