EXPERIMENTAL STUDIES ON THE DETECTION OF HYPNOTICS USING THIN LAYER CHROMATOGRAPHY

HISHIDA, Shigeru

Kobe J. Med. Sci. 18, 1-10, March 1972

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Shigeru HISHIDA, Mutsuko UEDA,
Takako TANABE and Yasuhiko MIZOI

Department of Legal Medicine,
Kobe University School of Medicine

Indexing Words
thin layer chromatography;
hypnotic;
development;
solvent system;
Rf value

Shigeru HISHIDA, Mutsuko UEDA, Takako TANABE and Yasuhiko MIZOI *Experimental Studies on the Detection of Hypnotics Using Thin Layer Chromatography.* Kobe J. Med. Sci. 18, 1-10, March 1972——The examination of hypnotics in the cadaver material frequently becomes the daily task in legal medicine and toxicology. In the present study, therefore, the use of thin layer chromatography for the identification of hypnotics was studied.

For the purpose of development of the drugs on the thin layer plate, four kinds of solvent system, viz. Acetone/Chloroform=1/9, Piperidine/Petrolether=1/5, Isopropylalcohol/Ammonia/Chloroform=45/10/45 and Chloroform/Methanol/Acetic acid=95/5/1 were used.

It is possible to identify eight kinds of hypnotics, namely barbiturates, bromine contained ureides, gultarimide and quinazolone, after finding the characteristic of Rf values of each of them by using the above mentioned four solvent system. In addition, it is also able to separate and detect these drugs simultaneously after mixing them in various proportion.

The similar results were attained, in the detection of drugs from stomach contents of dead bodies and from the blood, urine and organs in experimental animals. From these results, it was confirmed that the thin layer chromatography has wide application for practical use in the fields of legal medicine and toxicology.

INTRODUCTION

Nowadays quite many kinds of hypnotics are on the market and suicide and poisoning caused by hypnotics have remarkably increased. Therefore, it has become a very important problem to detect hypnotics in the corpse and biological material from the viewpoint of forensic toxicology.

For detection and identification of hypnotics, the color reaction method, crystallized method and spectrophotometric method have been so far used, but in each method it is absolutely necessary to use highly pure specimen, in order to ensure accuracy of detection. However, it has been extremely difficult to obtain specimen of high purity from all available materials despite the strenuous efforts made by Dressler[3] and Last[12].

Received for publication January 24, 1972.
Authors' names in Japanese: 菱田繁, 植田睦子, 田辺隆子, 溝井泰彦

With the spectrophotometric method, many reports have dealt with the results of detection of barbiturates in blood and tissue but accurate detection was found to be in extremely diffficulty in many practical cases due to various foreign substances containing the extracts. In addition, it is impossible to detect particularly some kinds of nonbarbiturates in the absence of the special absorption curve[9,20].

The paper chromatography has been most popular for some times past but this method has a defect that it takes quite a long time in the process of analyses. The absorption chromatography causes diffficulty in the process of filling the absorbent in the column to attain a certain analytical condition and moreover, it is impossible to run the accompanying material together by the same method.

For solution of these defects, Stahl[17] has devised "Thin Layer Chromatography" as one of the open column chromatography.

Thin layer chromatography made it possible to identify the various drugs and its metabolites by choosing the solvent system for development or the spray reagent[6, 14, 15, 18].

In this paper we deal with the analytical method for eight hypnotics which are most common on the market in our country, by developing both single and mixed hypnotic and for the samples obtained from the experimental animal on the thin layer plate with four different solvent systems.

MATERIALS AND METHODS

(1) *Thin layer plate:*

The thin layer plates were prepared according to Stahl's method, and 30 g of silica gel GF 254 was mixed by stirring in approximately 60 ml of distilled water and quickly applied to the five glass plates of 20×20 cm in one stroke. The glass plates were thus coated with a layer of standard thickness of 0.25 mm. The activation of the plates was peformed at 110°C for 30 min.

(2) *Samples:*

Three kinds of barbiturates, namely, Phenobarbital, Barbital and Adorm (Phanodorm), two kinds of bromine contained ureides, i. e. Brovarin (Bromural) and Adalin, carbamate (Valamin), gultarimide (Doriden) and quinazolone (Hyminal) were

Table 1. Eight groups of mixture sample

Number of mixture	Name of drugs
No. 1	Phenobarbital Barbital Adorm
No. 2	Brovarin Adalin
No. 3	Valamin Doriden Hyminal
No. 4	Phenobarbital Barbital Brovarin Adalin
No. 5	Barbital Brovarin Valamin Doriden
No. 6	Adorm Adalin Doriden
No. 7	Adorm Brovarin Hyminal
No. 8	Phenobarbital Barbital Adorm Brovarin Adalin Valamin Doriden Hyminal

used as samples.

Eight kinds of hypnotics were prepared as 1% chloroform solution respectively and 2-5 μl of these solutions which contained 20-50 μg of the drugs were spotted on the coated plate simultaneously with a microsyringe. On the other hand, these solutions were mixed in the same volume and made eight groups of mixture samples, No. 1-No. 8, as shown in Table 1. The 2-5 μl of the mixture sample solution which contained 10 μg of each drug was also spotted.

(3) *Analytical procedure:*

Four different solvent systems were used for developing; these were A-Chloroform/Acetone=9/1, B-Piperidine/Petrolether=1/5, C-Isopropylalcohol/Ammonia/Chloroform=45/10/45 and D-Chloroform/Methanol/Acetic acid=95/5/1.

The development distance was 10 cm and the time for each test was within 30 minutes. Desaga's test mixture was developed simultaneously with each hypnotic. For the detection of the drugs, the thin layer plates were sprayed with a 1% mercurous nitrate solution, and a few minutes later 0.04% Fluorecein reagent was added.

(4) *Animal experiments:*

Rabbits, weighing about 2.5-3.0 kg were used in the experiment. Each of eight hypnotics was poured into the rabbit stomach at a rate of 500 mg per kilogram through a rubber tube with 10-20 ml water. After 30 minutes, one hour and five hours, respectively, the rabbits were bled to death by cutting the carotid artery. Immediately after the death of the rabbits, 50 ml of blood, 10-150 ml of urine, 50g of liver, 10 g of brain and 20 g of kidney were obtained as the specimen and the Stas-Otto's extraction procedure was applied to them. The specimens were spotted on the thin layer plates as chloroform solution and developed with four kinds of solvent system.

RESULTS

A *Fundamental experiments*

(1) *Rf values of eight hypnotics*

The above mentioned single samples of the eight hypnotics were developed with four solvent systems and the results are shown in Figure 1.

In solvent system A, three kinds of barbiturate and Brovarin made the height of spots the same, while, Adalin, Valamin, Doriden and Hyminal showed the spots at different heights respectively, though the difference was minor. In solvent system B, all hypnotics made the height of spots different. In solvent system C, barbiturates and nonbarbiturates were clearly separated. Further, the three barbiturates which were difficult to be distinguished from the other three solvent systems were separated. In solvent system D, the three barbiturates rose to the same height, but the other drugs made the height of spots different.

The averages of Rf value obtained from the twenty times experiments using each solvent are shown in Table 2. The results of the accurate Rf values of hypnotics were obtained under the same analytical conditions. The fluctuation of Rf value was hardly observed in the solvent system A, C and D. In the solvent system B, minor fluctuation

was seen with three barbiturates, but with nonbarbiturates some fluctuation was observed.

Table 2. Rf values of eight kinds of hypnotics

Drugs	Solvent system A	B	C	D
Phenobarbital	0.19 (± 0.03)	0.10 (± 0.02)	0.31 (± 0.03)	0.32 (± 0.03)
Barbital	0.18 (± 0.03)	0.21 (± 0.04)	0.40 (± 0.04)	0.30 (± 0.02)
Adorm	0.20 (± 0.03)	0.15 (± 0.02)	0.45 (± 0.04)	0.31 (± 0.02)
Brovarin	0.22 (± 0.04)	0.55 (± 0.07)	0.87 (± 0.04)	0.36 (± 0.04)
Adalin	0.38 (± 0.06)	0.70 (± 0.07)	0.92 (± 0.04)	0.57 (± 0.05)
Valamin	0.35 (± 0.05)	0.44 (± 0.04)	0.88 (± 0.04)	0.47 (± 0.04)
Doriden	0.46 (± 0.05)	0.81 (± 0.06)	0.93 (± 0.04)	0.65 (± 0.05)
Hyminal	0.52 (± 0.08)	0.78 (± 0.06)	0.93 (± 0.03)	0.75 (± 0.03)
Test solution	0.71	0.75	0.91	0.82

( ): S. D.

(2) *Separation of hypnotics in mixed solution*

The separation of the two to eight kinds of hypnotics in mixture samples was performed by using the solvent system A to D as developing solution.

The chromatogram of the spots obtained is shown in Figure 2. In the solvent system A, the values of barbiturates and Brovarin were nearly equal and they were recognized almost in one spot, which was impossible to be differentiated, whereas Valamin, Adalin, Doriden and Hyminal were perfectly distinguished from one another due to their different Rf values. On the other hand, with the solvent system B, each spot of the drugs was distinctly separated in all mixture samples, and was clearly recognized even in sample No. 8, in which all of the eight kinds of hypnotics were contained. In the solvent system C, barbiturates and nonbarbiturates were separated distinctly as described above, and the spots of three kinds barbiturates showed only one spot, but non bar-biturates were recognized to be separated distinctly.

Conspicuous differences of Rf values of the hypnotics were not found in the both experiments in which single and mixture samples were used (Fig. 3).

(3) *Separation of hypnotics mixed into stomach contents*

Firstly, 100 mg of eight drugs each was miexd into approximately 200 ml of stomach contents and then the hypnotics were extracted as the ether solution by Stas-Otto procedure. These extracts, after the evaporation of ether, were redissolved in chloroform and then developed with the solvent system A, B, C and D.

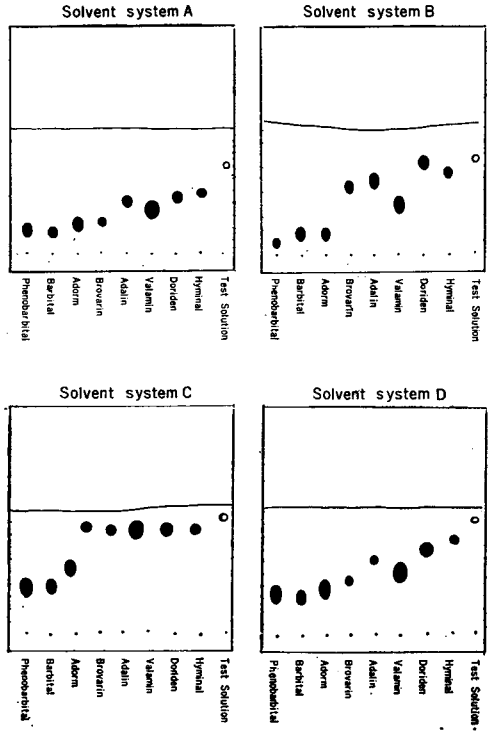

Fig. 1. Thin layer chromatogram of eight kinds of hypnotics. Solvent system A: Chloroform/Acetone=9/1, B: Piperidine/Petroleum ether=1/5, C: Isopropyl alcohol/Ammonia/Chloroform=45/10/45, D: Chloroform/Methanol/Acetic acid=95/5/1.

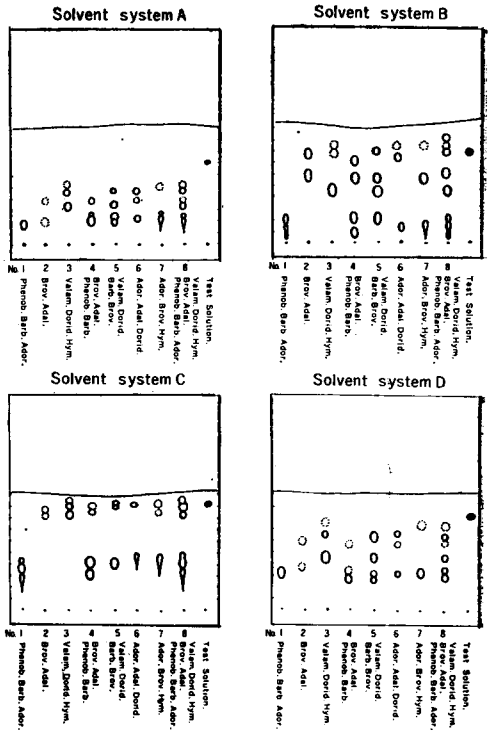

Fig. 2 Thin layer chromatogram of eight kinds of mixture samples. Solvent system, A: Chloroform/Acetone=9/1, B: Piperidine/Petroleum ether=1/5, C: Isopropyl alcohol/Ammonia/Chloroform=45/10/45, D: Chloroform/Methanol/Acetic acid=95/5/1. Development distance: 10 cm.

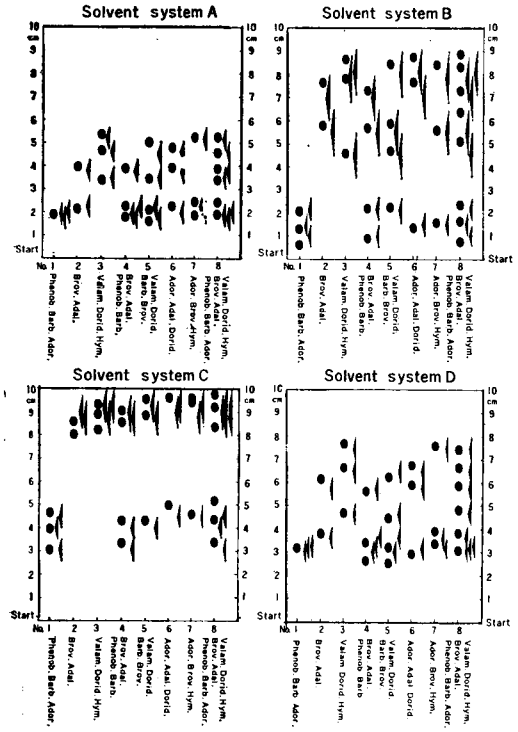

Fig. 3 Shematic chromatogram of mixture samples (round spots) in comparision with the variation of Rf values when single samples were developed (hill-shaped marks).

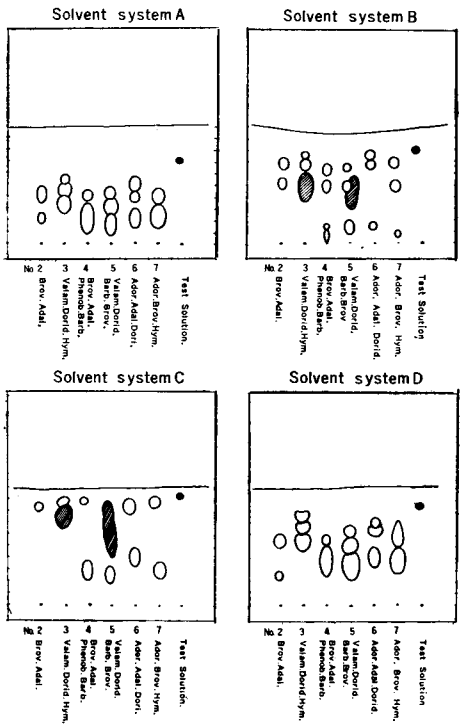

Fig. 4 Schematic chromatogram of the extracts from stomach contents mixed with mixture samples [No. 2 to No. 7. Solvent system, A: Chloroform/Acetone=9/1, B: Piperidine/Petroleum ether=1/5, C: Isopropyl alcohol / Ammonia / Chloroform= 45/10 / 45, D: Chloroform / Methanol / Acetic acid=95/5/1, Development distance 10 cm.

Table 3. Rf values of the drugs mixed in the stomach contents

Drugs	Solvent system A	B	C	D
Phenobarbital	0.19 (0.19)	0.09 (0.10)	0.36 (0.31)	0.33 (0.32)
Barbital	0.19 (0.18)	0.17 (0.21)	0.37 (0.40)	0.30 (0.30)
Adorm	0.23 (0.20)	0.17 (0.15)	0.53 (0.45)	0.33 (0.31)
Brovarin	0.27 (0.22)	0.53 (0.55)	0.83 (0.87)	0.43 (0.36)
Adalin	0.44 (0.38)	0.58 (0.70)	0.81 (0.92)	0.60 (0.57)
Valamin	0.37 (0.35)	0.40 (0.44)	0.82 (0.88)	0.53 (0.47)
Doriden	0.46 (0.46)	0.73 (0.81)	0.84 (0.93)	0.68 (0.65)
Hyminal	0.50 (0.52)	0.67 (0.78)	0.84 (0.93)	0.74 (0.75)
Test solution	0.71	0.75	0.92	0 87

Figures in parentheses indicate Rf value of single samples.

Table 3 shows the Rf values of the hypnotics thus obtained in comparison with the Rf values obtained by single sample, and the both Rf values did not show any difference. Likewise, the Rf values obtained by developing the extracts from stomach contents mixed with mixture samples No. 2-No. 7 are shown in Figure 4. These Rf values were compared with those of single pure samples and any significant difference was not recognized between them.

B *Experiments with rabbits*

Generally, the symptoms such as snoring sleep and atony of extremties were clearly observed about one hour after intake of hypnotics. Each hypnotic was detected in blood 30 min after administration but was not detected in urine. After one hour, hypnotics were detected successfully in all of the materials. After five hours, hypnotics were detected more conspicuously in urine than in any other organs and further, spots which could be recognized as metabolites of hypnotics were also observed.

DISCUSSION AND CONCLUSIONS

In the field of forensic toxicology thin layer chromatography (TLC) was adopted, for the first time, for detection of morphine, codein and narcotine in the stomach contents, and the other various drugs[13]. Frhan et al[5] has detected 15 kinds of hypnotics in urine by using solvent system of Isopropylalcohol, Ammonia and Chloroform, and Jansch[11] has successfully detected Amytal and Seconal in the stomach contents, blood and urine of a dead body of Perdomal poisoning. Using Piperidine-Petrolether solvent system, Ederhard et al[4] examined the metabolites in the urine

of patients who had taken four kinds of barbiturates and eight kinds of non-barbiturates.

In the case of identification of various hypnotics by means of TLC especially if different kinds of hypnotics are mixed, the detection of each hypnotic with only one solvent system becomes extremely difficult, because TLC dose not necessarily show the specific Rf values. In addition, the Rf values fluctuate depending on the change of analytical condition. Some investigators have tried to identify hypnotics by means of color reaction method using spray reagent, but generally this method is not so suitable by reasons of possible misjudgment due to the foreign substance contained hypnotics [1, 2, 7, 8, 16, 19].

For these reasons, it is advisable to compare the Rf values obtained with two or four different solvent system, if possible, so that more accurate identification may be expected.

In the present studies, four solvent systems were used as developer and at the same time, the test mixture was developed considering the changes in analytical condition. Consequently the specific Rf values of each hypnotic have been obtained as shown in Figure 5. The fluctuations of Rf values were rarely seen if the same conditions of development were applied.

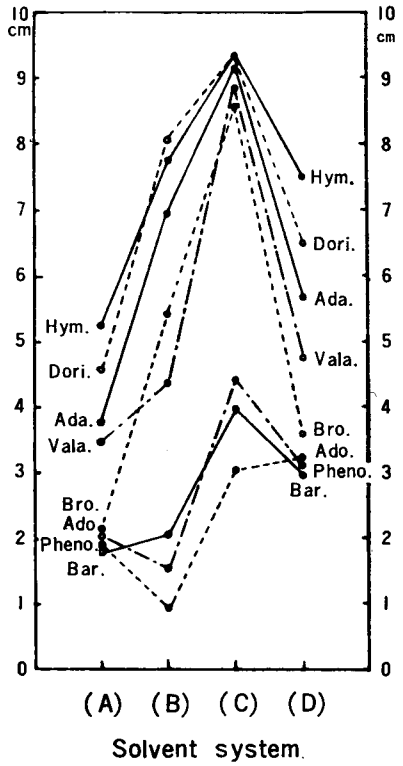

Fig. 5 Relationship of Rf-values of each hypnotic by using four kinds of solvent system.

When more than two kinds of hypnotics were mixed, the solvent system B showed a comparatively high separation ability, but it is quite difficult to separate and detect hypnotics with solvent system B only because of the conspicuous fluctuations of Rf values.

It becomes possible to separate and detect hypnotics exactly by developing the drugs according to the following method with the four different solvent system A, B, C and D. In the detection of unknown hypnotics—(i) barbiturates and nonbarbiturates are separated by developing with the solvent system C and (ii) the hypnotics are identified according to the Rf values obtained by developing with the solvent system A, B and D.

This method has been succeeded with the extracts from stomach contents and the blood, urine and organs(namely, liver, brain and kindey) obtained in animal experiments. We have so far confirmed that TLC has many advantages such as (a) hypnotic can be detected even in rough extracts, (b) the time required for detection is short, and (c) its good sensitivity made it possible to detect with only a small amount of specimen. Furthermore, taking advantage of the good separation ability of the TLC method, we could make a quantitative analysis of pure eluated material separated by the same method[10].

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