On the Separation and Identification of Sulfonamides by the Thin-Layer Chromatography and the Detection of Sulfa Drug in the Cadaveric Blood

Junji FURUNO, Norisuke SUGAWARA and Takeshi KAN

Department of Legal Medicine, Yamaguchi University School of Medicine, Ube

(Received July 13, 1969)

Accompanied with the development of a remidy, the drug treatment has been remarkably progressed. On the contrary, the fatal cases on account of the shock with medicines have now showed an increasing tendency. From thus situation the forensic toxicologist should always keep such cases in mind and do the studies on the simultaneous analysis over a great number of medicines.

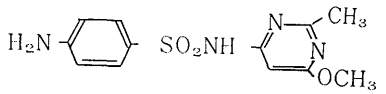
For sulfonamides the simultaneous analysis has been already reported through the paper partition[1-3] and thin-layer chromatography by some workers. Especially in the thin-layer chromatography the following works have been seen up to date; namely, Wollish[4] have chromatographied five kinds of sulfonamides using a mixture of chloroform-pentane-ethanol (1 : 1 : 1) as mobile phase, Klein et al.[5] attempted the analysis for their various kinds employing the characteristic plate, and Fuwa et al.[6] carried out the studies on the separation and detecting reagents for their fourteen kinds. Whereas, the satisfactory separation would not be expected yet.

Recently, a man aged fifty two had suddenly died after the intravenous injection of sulfa drug, was subjected to the pathological anatomy, and we were entrusted with tests of it in his blood. So, in addition to this we have carried out the systematic studies for the separation and identification of seven kinds of sulfonamides well used in common by the thin-layer chromatography.

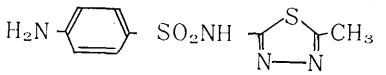
MATERIALS AND METHODS

1) Materials

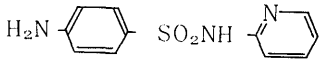
Sulfamethomidine (Tanabe)



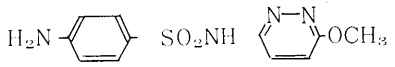
Sulfamethizole (Ezai)



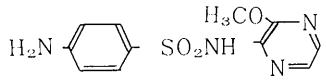
Sulfapyridine (Tanabe)



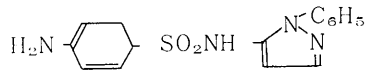
Sulfamethoxypyridazine (Takeda)



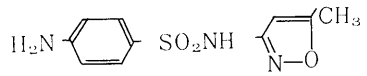
Sulfamethopyrazine (Ezai)



Sulfaphenazole (Dainippon)



Sulfaisomezole (Shionogi)



2) Preparation of materials

Sulfonamides each was dissolved in acetone and adjusted to the concentration of 2 μg/μl.

3) Preparation of plate

Thirty g of silica gel G (Merk) was placed in a motor. To this was added 60 ml of deionized water and the mixture was rigorously stirred until uniform. The slurry was poured onto the glass plate (20×20 cm) and spread uniformly with a rod of glass equipped with the cellophane tape of 250 mμ in thickness to one's both ends. After drying in air, the plate was left in an oven at 110±10°C 1 hour and kept in a box containing silica gel desiccant.

4) Developers

Developer A[5]: Chloroform-heptane-ethanol (1 : 1 : 1). To this mixture, deionized water was added to give the concentration of 1.2 %.

Developer B[1]: Fifty ml of n-butanol was mixed with 15 ml of glacial acetic acid and 60 ml of deionized water. The mixture was well shaken in a separating funnel and allowed to separate. The lower aqueous layer was discarded and p-dimethylaminobenzaldehyde added to the remaining solvent to give a concentration of approximately 0.5 %.

Developer C[1]: Forty ml of n-butanol was mixed with 10 ml of conc. ammonia water and 30 ml of deionized water. The mixture was shaken up as with the acid solvent, separated and p-dimethylaminobenzaldehyde added to the organic layer to give a concentration of approximately 0.5 %.

Developer D: n-Butanol-deionized water-conc. ammonia water (100 : 66 : 33)

Developer E[7]: Chloroform-methanol (80 : 15)

Developer F: Chloroform-methanol (70 : 30)

Developer G: n-Butanol-heptane-ethanol (1 : 1 : 1)

Developer H: Chloroform-methanol-acetone (80 : 15 : 15)

Developer I: n-Butanol-methanol-acetone (80 : 15 : 15)

Developer J: n-Heptane-methanol-acetone (80 : 15 : 15)

Developer K: Eighty ml of chloroform, 15 ml of methanol, 15 ml of acetone, and 15 ml of deionized water were mixed. The mixture was well shaken in a

separating funnel and allowed to separate. The upper aqueous phase was discarded and the lower chloroform phase employed.

Developer L : Dichloromethane-methanol-acetone (80 : 15 : 15)

Developer M : Chloroform-acetone-methanol-glacial acetic acid (80 : 10 : 10 : 10)

Developer N[8] : Cyclohexane-methanol-dimethylamine (60 : 30 : 10)

Developer O : Chloroform-methanol-acetone-conc. ammonia water (80 : 15 : 15 : 5)

5) Developing procedure

The original solution (5 μl) was spotted onto the adsorbent at a point of 2 cm from one edge of the plate. The development was carried out in an atomosphere saturated with respect to a developer by one dimentional ascending method. Except one part the developed distance is 10 cm.

6) Detecting reagents

Ehrlich's reagent : Two g of p-dimethylaminobenzaldehyde was dissolved in 100 ml of 6 % hydrochloric acid.

Diazo reagent : Two N hydrochloric acid, 2 % sodium nitrite, and 0.02 % N-ethyl-α-naphthylamine (in ethanol) each was prepared. On color, sulfonamides on the plate were diazonized by spraying hydrochloric acid, followed by sodium nitrite and at latest the diazonium salt was coupled, by spraying, with N-ethyl-α-naphthylamine to color.

7) Apparatus

The ultrviolet and infrared absorption spectra were recorded on Hitachi-Perkin Elmer ultraviolet spectrophotometer Model 139 and Nipponbunko infrared spectrophotometer Model IRS, respectivly.

EXPERIMENTAL RESULTS AND DISCUSSION

1) Studies on developers

The authors have attempted the simultaneous analysis of seven kinds of sulfonamides using various mixtures which were new prepared to pay an attention on the quality of solvent, e. g., acidity or alkalinity and polarity etc. and besides, those reported already in the references as regards the paper partition and thin-layer chromatographic analysis of sulfonamides. The Rf values and chromatograms are shown in Table 1 and Photos. 1–3, respectively.

Table 1. Rf value of seven kinds of sulfonamides in thin-layer chromatography

Developer / Sample	Rf value														
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
Sulfamethomidine	0.59	0.78	0.37	0.37	0.71	0.80	0.55	0.53	0.73	0.11	0.38	0.72	0.82	0.44	0.07
Sulfamethizole	0.37	0.80	0.32	0.31	0.39	0.53	0.27	0.26	0.58	0.12	0.14	0.82	0.69	0.37	0.02
Sulfapyridine	0.54	0.72	0.44	0.40	0.64	0.74	0.52	0.55	0.80	0.11	0.34	0.72	0.72	0.38	0.02
Sulfamethoxypyridazine	0.65	0.74	0.33	0.28	0.75	0.81	0.57	0.49	0.76	0.11	0.41	0.77	0.73	0.44	0.09
Sulfamethopyrazine	0.69	0.78	0.30	0.25	0.84	0.81	0.57	0.68	0.80	0.11	0.58	0.84	0.79	0.41	0.07
Sulfaphenazole	0.69	0.83	0.62	0.53	0.76	0.81	0.64	0.61	0.86	0.11	0.50	0.85	0.76	0.41	0.07
Sulfaisomezole	0.68	0.83	0.32	0.29	0.74	0.78	0.62	0.57	0.83	0.08	0.44	0.48	0.75	0.39	0.04

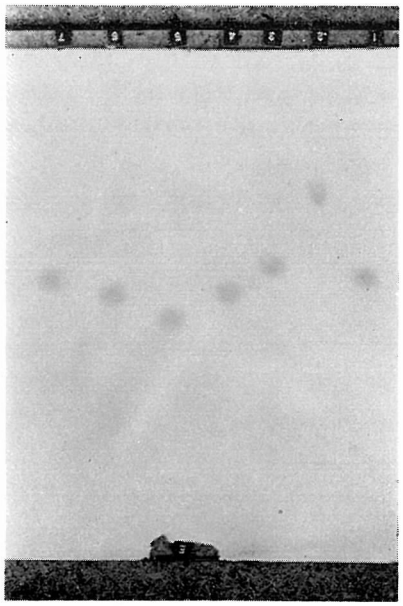


Photo 1. Thin-layer chromatograms in the use of developer E
1: Sulfamethomidine
2: Sulfamethizole
3: Sulfapyridine
4: Sulfamethoxypyridazine
5: Sulfamethopyradine
6: Sulfaphenazole
7: Sulfaisomezole

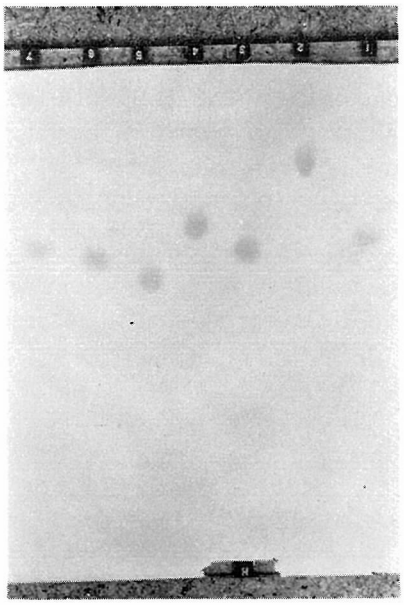


Photo 2. Thin-layer chromatograms in the use of developer H

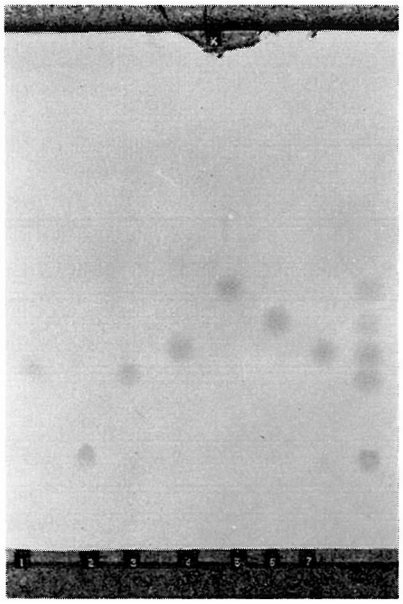


Photo 3. Thin-layer chromatograms in the use of developer K

Of the developers used, the developers of E, H, and K were more excellent than the others in the separation of these sulfonamides. In especial the K was the most satisfactory and as shown in Table 1, the Rf values in this solvent were as followed ; sulfamethomidine 0.38, sulfamethizole 0.14, sulfapyridine 0.34, sulfamethoxypyridazine 0.41, sulfamethopyrazine 0.58, sulfaphenazole 0.50, sulfaisomezole 0.44. Thus, inasmach as sulfamethomidine, sulfamethoxypyridazine, and sulfaisomezole were closely related in their Rf values, they were difficult to separate. But among either of these sulfonamides and four others, viz., sulfamethizole, sulfapyridine, sulfamethopyrazine, and sulfaphenazole, the separation was entirely achieved. Furthermore, it is worth for note that the good developers mentioned above, E, H, and K each consist of two or more kinds of polar and non-polar solvents, being of chloroform-methanol system.

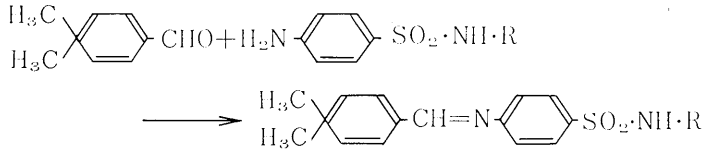
Both the developer B and C, proposed by Robinson[1] are differentiated from all others in the constitution of solvent. The former is the upper layer of a mixture of n-butanol-glacial acetic acid-deionized water (50 : 15 : 60), the later is the organic layer of a mixture of n-butanol-conc. ammonia water-deionized water (40 : 10 : 30), and both are characteristic, especially in point of containing p-methylaminobenzaldehyde of approximately 5 % by weight per volume. This may be assumed to be an attempt that in the course of development, allows NH_2-group of N^4-position of sulfonamides and CHO-group of p-dimethylaminobenzaldehyde condensately to react and separates sulfonamides in the form of their Schiff base. In the application of both developers to these sulfonamides the satisfactory separa-

tion could not be expected. Whereas, these developers make it possible to observe their chromatographic behaviors, because sulfonamides gradually reveal yellow spots during the development.

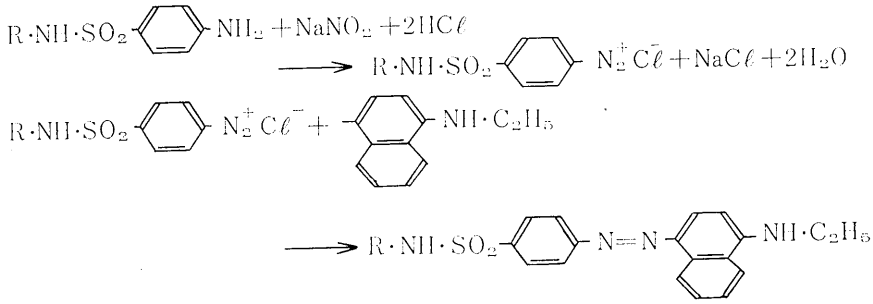
The relationship between the difference in the polarity of developer and the mobility of sulfonamide was not always discovered, but in cases of the mixtures made up with the same kinds of solvents, for example, in the developers of E and F (consisting of chloroform and methanol solvent), the Rf values in the later has a high content of methanol were more greater than those in the former. In the developer N, all the spots of sulfonamides used were divided into three branches, but in the others, such a troublesome tailing and spreading phenomenon of a spot was not observed at all.

2) Studies on detecting reagents

Each 5 μl of the acetone solution sulfonamides mention above (concentrations ranged from 0.1 to 10.0 μg/5 μl) was spotted onto the activated thin film and, by spraying both reagents of p-dimethylaminobenzaldehyde and diazo to this, the some studies were carried out on the color and the limit for detection. With both reagents all sulfonamides were detected in the weight order of 0.1 μg. The chromatograms of sulfonamides with p-dimethylaminobenzaldehyde reagent were yellow or orange yellow and those with diazo reagent pink or pink red, respectively. The color tone was very clear. The color reaction mechanisms of sulfonamides with both reagents are considered as follows. With p-dimethylaminobenzaldehyde reagent the color depends upon the formation of Schiff base in which the concentration of CHO-group of this reagent and NH_2-group of N^4-position of sulfonamide results.



In the case of diazo reagent, NH_2-group of N^4-position of sulfonamide allows to react with sodium nitrite under the presence of hydrochloric acid to form diazonium salt, and the diazonium salt is subsequently coupled with N-ethyl-α-naphthylamine to form the diazonium dye compound.



EXAMINATION OF MEDICO-LEGAL OBJECT

A healthy man aged 52 complained a slight fever, having a fever of 37.8°C, and consulted with a doctor in a certain hospital. Then, he was intravenously received "Daimeton" injection (volume is 10 ml; manufactured by Daiichiseiyaku K. K.), 10 minutes later became suddenly dyspnoe, and more 10 minutes later died. As described above, the blood was brought to our laboratory in order to test. A specimen was alone 8 ml of the blood. Ether was employed as an solvent for extracting by reason of being more less in the solubility of sulfamonomethoxine sodium, but immiscible in water. The blood was transferred into a separating funnel, to this was added about three times of ether. The mixture was well shaken, allowed to stand and ether phase was separate out. This procedure was repeated at least several times in order to get sulfamonomethoxine sodium as much quantities as possible. All the ether extract were combined and dried over anhydrous Na_2SO_4 and evaporated to dryness under the reduced pressure. The viscid, pale yellow residue was obtained. The residue could not be purified by the chromatography and recrystallization because of very small quantities. Subsequently, the standard samples, sulfamonomethoxine sodium and sulfamonomethoxine were extracted according to the following procedures. A "Daimeton" injection (5 ml; sulfamonomethoxine sodium and anhydrous sodium sulfite as a stabilizer are contained in the concentration of 10 and 0.2 w/v %, respectively) was employed as a material of sulfamonomethoxine sodium. It was evaporated to dryness under the reduced pressure and white residue was obtained. The residue was recrystallized three times from ethanol, m.p. 243–244°C.

The small quantities of this crystalline powder was dissolved in a little water, to this added a few drops of acetic acid. The white precipitate produced was collected by filteration, washed with deionized water, and recrystallized from ethanol, m. p. 204–206°C. And as a material of sulfamonomethoxine, "Daimeton" granules (993 mg of sulfamonomethoxine in 1 g of the granules) were used. Sulfamonomethoxine was extracted with acetone and the acetone extract was evaporated to dryness under the reduced pressure. The obtained crude crystals were recrystallized repeatedly from ethanol, m.p. 204–205°C. The standard samples (sulfamonomethoxine sodium and sulfamonomethoxine extracted by the method mentioned above) and blood extract each was dissolved in a small amount of ethanol, these solution were spotted onto the same plate, and the spots were developed a distance of 15 cm by oneway ascending method using both mixtures of chloroform-n-butanol-petroleum ether (1 : 1 : 1) and methanol-n-hexane (3 : 1). The plate was taken out from a development chamber, dried in air, and sprayed with p-dimethylaminobenzaldehyde or diazo reagent. In a mixture of chloroform-n-butanol-petroleum ether (1 : 1 : 1), the Rf values of sulfamonomethoxine sodium, sulfamonomethoxine and blood extract were 0.51, 0.45, and 0.51, and the Rf

values of those in a mixture of methanol-n-hexane (3 : 1) were 0.76, 0.74 and 0.75, respectively.

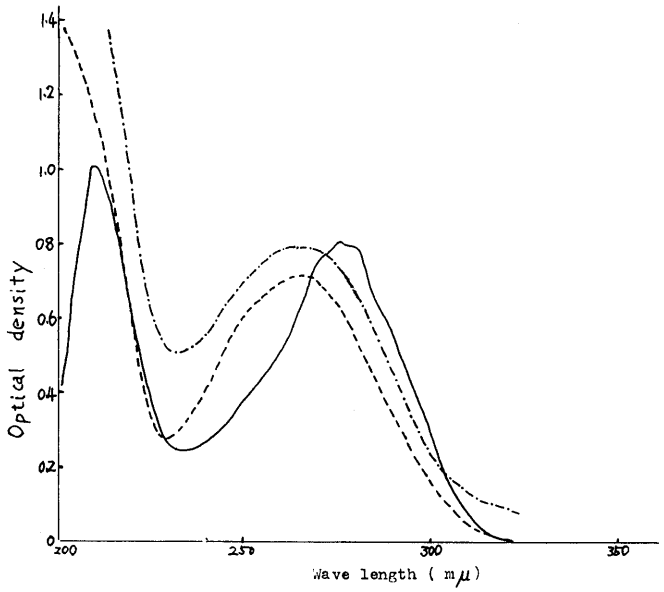


Fig. 1. Ultraviolet absorption spectra of sulfamonomethoxine (1), sulfamonomethoxine sodium salt (2) and blood extraxt (3)
——— 1 (in C_2H_5OH) ····· 2 (in H_2O) ···—··· 3 (in H_2O)

Further, for the purpose of raising the reliability of analysis, sulfamonomethoxine sodium and blood extract were dissolved in deionized water, sulfamonomethoxine in ether, and the ultraviolet absorption spectra of these solutions were measured (Fig. 1). Sulfamonomethoxine sodium had a absorption maximun at 264.5 mμ and a absorption minimum at 229 mμ, blood extract revealed a absorption maximum at 264.5 mμ and a absorption minimum at 232 mμ and sulfamonomethoxine gave a absorption maximum at 275 mμ and a absorption minimum at about 234 mμ. For reference, only the standard samples were subjected to the infrared absorption spectrophotometry (Fig. 2), but in case of blood extract it could not be carried out owing to the lack of quantities.

At latest, the remainder of blood extract after being supplied to the analysis by the thin-layer chromatography and ultraviolet absorption spectrophotometry, was dissolved in 20 ml of 3 % trichloroacetic acid, the precipitate was removed by centrifugation, and the supernatant was divided into two equal volumes. Ten ml of the one was directly put to the determination without pretreatment. Ten ml of the other was added 5 ml of 4 N hydrochloric acid and thus treated solution was boiled in a water bath for 1 hour and the total volume was adjusted to 10 ml with 3 % trichloroacetic acid. The determination was performed according to Bratton and Marshall's method[9] as follows.

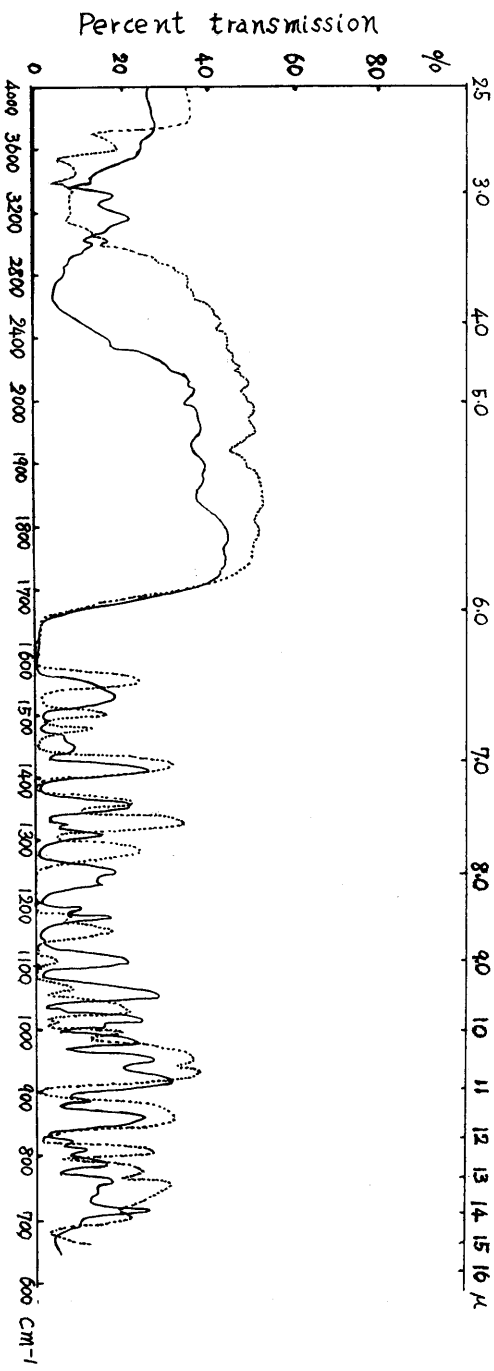


Fig. 2. Infrared spectra (KBr disc) of sulfamonomethoxine (1) and its sodium salt (2)
——— (1), ····· (2)

One ml of 1 % sodium nitrite was added to each 10 ml of both solutions. After 3 minutes standing, 1 ml of 0.5 % ammonium sulfamate was added and after a lapse of 2 minutes, 1 ml of 0.1 % solution of N-(1-naphthyl) ethylendiamine dihydrochloride was added. The diazo compound formed was measured at 530 mμ. The ultraviolet absorption spectra at the range of wave lengths from 500 to 580 mμ is shown in Fig. 3 and the calibration curve in Fig. 4, respectively. On the basis of these measured values, the regression curve, $Y=1.102X-0.003$, where X is the concentration of sulfamonomethoxine sodium (mg %) and Y the

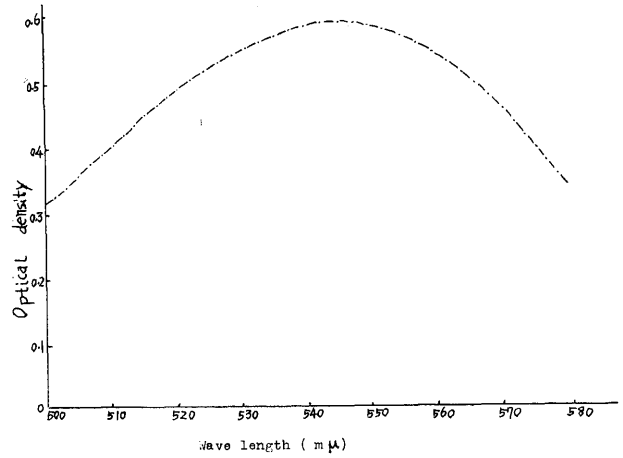


Fig. 3. Ultraviolet absorption spectra of sulfamonomethoxine diazo compound

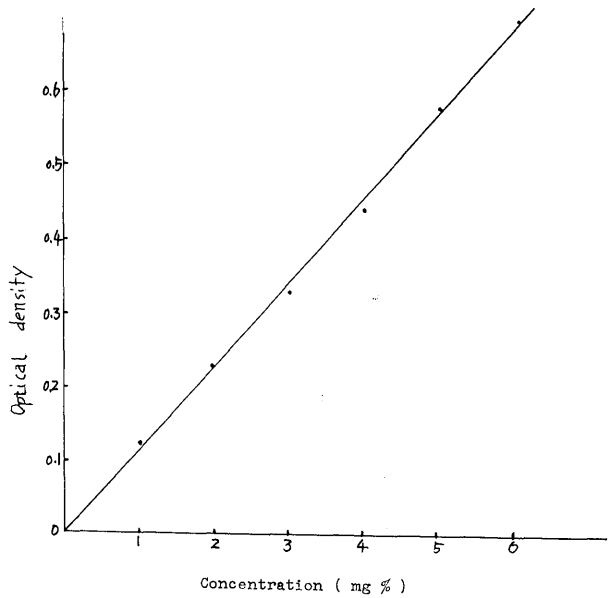


Fig. 4. Calibration curve of sulfamonomethoxine sodium salt

optical density (−log T) was obtained. The quantities of sulfamonomethoxine sodium were 1.6 mg in the non-hydrolyzed blood extract and 1.5 mg in the hydrolyzed, respectively. In a fact that the result for determination of the former closely agreed with that of the later, the metabolites of sulfonamides detected frequently under the administration of sulfa drugs, viz., N^4-acetylate, N^4-sulfonate, N^4-glucronide etc.[10)11)] may be assumed not to exist in the blood extract.

SUMMARY

The authors have described the separation and identification of seven kinds of sulfonamides by means of the thin-layer chromatography and an example for the detection of sulfamonomethoxine sodium in the cadaveric blood.

In the separation of these sulfonamides, the developers of chloroform-methanol system which consist of two or more kinds of polar and non-polar solvents, gave the satisfactory result. All these sulfonamides could be detected even in a very small quantities of 0.1 μg by using p-dimethylaminobenzaldehyde or diazo reagent and the color was very clear. As a result, both reagents would be expected to be available for the detecting reagent in the usual analysis of sulfonamides.

From the blood (8 ml) of a man aged 52 who "Daimeton" injection was intravenously received and suddenly died, sulfamonomethoxine sodium was separated and identified through the analysis coupled with the thin-layer chromatography and ultraviolet absorption spectrophotometry. The quantities of sulfamonomethoxine sodium determined about the remainder of blood extract after the qualititive analysis was approximately 3.0 mg.

REFERENCES

1) Robinson, R.: *Nature*, **168**: 512, 1951.
2) Longnecker, W. H.: *Anal. Chem.*, **21**: 1502, 1949.
3) Thomas, G. et al.: *J. pharm. Berg.*, (2), **5**: 263, 1950; *C.A.* **45**: 4406-f, 1951.
4) Wollish, E. G.: *Anal. Chem.*, **33**: 1138, 1961.
5) Klein, S. et al.: *J. pharm. Science*, **51**: 966, 1962.
6) Fuwa, T. et al.: *Yakuzaigaku*, **23**: 102, 1963.
7) Stall, E.: *Dünnschicht-chromatographie*, 323, Sppinge-Verlag, 1962.
8) Stall, E.: *ibid*, s. 324.
9) Bratton, A. C. et al.: *J. Biol. Chem.*, **128**: 537, 1939.
10) Uno, T. et al.: *Yakugaku Zassi*, **82**: 759, 1962.
11) Uno, T. et al.: *Chem. Pharm. Bull.*, **11**: 872, 1963.