Journal of Chromatography, 155 (1978) 223–225

CHROM. 10,955

Note

Thin-layer chromatographic procedure for the quantitative assay of NH-containing compounds

G. PREISSER and U. DEMME
Institut für gerichtliche Medizin, Friedrich-Schiller-Universität, 69 Jena (G.D.R.)
(First received August 31st, 1977; revised manuscript received February 15th, 1978)

The application of chlorine with subsequent detection with benzidine or *o*-tolidine as a reagent for proteins and amino acids on paper chromatograms was investigated by Rydon and Smith[1] and by Reindel and Hoppe[2]. The method is generally applicable to the detection of NH-containing substances such as primary and secondary amines or N-acylated nitrogen compounds.

The reaction of the chloramines and chlorimides formed by chlorination with benzidine or *o*-tolidine leads to dyestuffs with different colours and intensities of colour[2], so that elution and subsequent spectrophotometric determination are therefore generally not possible. The use of potassium iodide solution results in the generation of more intensively coloured compounds but they are almost insoluble in water and organic solvents and are therefore difficult to elute from thin-layer chromatographic (TLC) plates.

Therefore, we tried to reveal the spots of these substances by omitting the benzidine or *o*-tolidine and spraying the plates only with potassium iodide solution, and then eluting the iodine generated for subsequent reaction with a suitable reagent in aqueous solution. At first these attempts failed because the iodine generated was too volatile to yield reproducible results. However, the application of a solution of potassium iodide in ethylene glycol produced an improvement, and N-dimethyl-*p*-phenylenediamine was a suitable reagent for reaction with the iodine[3]. The intensity of the colour is high and the colour is sufficiently stable.

EXPERIMENTAL

Reagents

The thin-layer plates were coated with silica gel (Kieselgel G; Merck, Darmstadt, G.F.R.) to a thickness of 0.25 mm. The chlorine was taken from a pressurized bottle without further purification. When it is prepared from potassium permanganate and hydrochloric acid, it should be dried with calcium chloride.

The ethylene glycol was of the highest grade available and a 3% solution of potassium iodide was prepared in it. The solution must not contain free iodine (brown colour) or consume iodine when 0.01 *N* iodine solution is added. If these conditions are not met, 0.1 *N* thiosulphate or 0.01 *N* iodine is added. The solution should be kept in a refrigerator and checked weekly.

The colour reagent consisted of a 0.5% solution of N-dimethyl-*p*-phenylenediamine in ethanol and was kept in a refrigerator. It must be renewed if it becomes strongly discoloured.

All other solvents were analytical-reagent grade materials.

Procedure

An aliquot of the solution, containing up to 30 μg of the compound under investigation, is applied to the TLC plate with a μl-syringe, together with several standards, *e.g.*, 10, 20 and 30 μg. One position at the start is left untreated and acts as a blank at the corresponding R_F value. The plate is developed with a suitable solvent, which should be slightly volatile, *e.g.*, chloroform, acetone, diethyl ether, methanol, ethanol of their mixtures. Solvent systems containing ammonia or other bases cannot be used, because these cannot be removed from the plate even when it is heated. In such instances very high blanks are obtained.

After development, the plate is dried by heating for 10 min. After cooling to room temperature, the plate is chlorinated in a chamber saturated with chlorine for 1 min, then the excess of chlorine is removed by passage of a stream of cold air for 1 min. The plate is then immediately sprayed with the solution of potassium iodide in ethylene glycol until the layer is just visibly moistened. The spots, including the blank, were circled in equal areas and the layer was scraped off quantitatively with a sharp spatula and transferred into a centrifuge tube containing 3 ml of water. When the spray is applied correctly, the layer can easily be removed in the form of chips. This step must be completed within a few minutes in order to prevent losses of iodine. When the iodine-containing silica gel has been transferred into the water, the time is less important and the further treatment can be carried out during the next few hours.

After mixing the contents by hand, the tubes are centrifuged for 3 min at 500 *g* and the clear supernatant is decanted into another tube. Immediately before measurement 0.2 ml of N-dimethyl-*p*-phenylenediamine is added and the samples are read against the blank at 555 nm in 1-cm cells. The colour fades slowly at a rate of about 1–2% per 5 min.

A calibration graph is drawn and experimental concentrations are read off in the usual manner. To test the reproducibility and precision of the method, four independent analyses on different thin-layer plates were carried out and the results are presented in Table I. The application of standards on each plate is necessary in order to compensate for differences in the blank and in the extent of chlorination.

The time needed for the handling of one plate, including application, chlorination, elution and measurement, is *ca.* 45 min.

RESULTS AND DISCUSSION

Fig. 1 shows examples for calibration graphs for different compounds. The graphs are linear up to at least 30 μg. The minimal concentration distinguishable from the blank is about 2 μg or less.

The different slopes of the calibration graphs depend mainly on the different NH contents as a function of molecular weight. Moreover, in some instances the influence of the functional groups adjacent to the NH group can be discerned, especially with barbiturates, where the NH group is more acidic.

TABLE I

ASSESSMENT OF THE REPRODUCIBILITY AND PRECISION OF THE METHOD

Results are means ± standard deviations ($n = 4$)

Substance determined	*Concentration applied (μg)*		
	10	*20*	*30*
Glycine ethyl ester	10.0 ± 0.3	20.6 ± 0.5	29.8 ± 0.3
Glycine	10.1 ± 0.3	19.9 ± 0.8	29.5 ± 0.3
Hippuric acid	10.0 ± 0	19.9 ± 0.7	29.2 ± 0.3
Acetanilide	10.0 ± 0.3	20.6 ± 0.4	30.1 ± 0.4
Diphenylamine	10.0 ± 0.6	20.6 ± 0.5	30.1 ± 0.7
Anisidine	9.9 ± 0.4	20.0 ± 0.3	29.1 ± 0.4
Dimethylpropanedioldicarbamate	10.0 ± 0.5	19.6 ± 0.7	30.3 ± 0.4
Phenobarbitone	10.4 ± 0.4	20.2 ± 0.3	30.0 ± 0.6

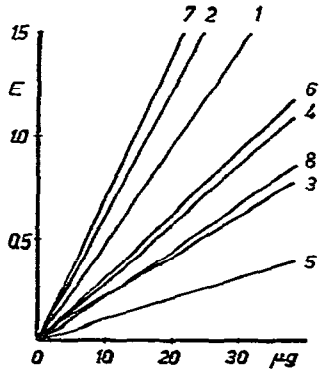

Fig. 1. Examples of calibration graphs. 1 = Glycine ethyl ester; 2 = glycine; 3 = hippuric acid; 4 = acetanilide; 5 = diphenylamine; 6 = anisidine; 7 = dimethylpropanediol dicarbamate; 8 = phenobarbitone.

The much lower extinction with diphenylamine is caused by side-reactions, because the spots on the plate become coloured as a result of chlorination.

ACKNOWLEDGEMENTS

The authors thank Mrs. H. Steil and Mrs. R. Meixner for their excellent technical assistance.

REFERENCES

1 H. N. Rydon and P. W. Smith, *Nature (London)*, 168 (1952) 922.
2 F. Reindel and W. Hoppe, *Chem. Ber.*, 87 (1954) 1103.
3 A. T. Palin, *Analyst (London)*, 70 (1945) 203.