Separation and detection of oral hypoglycaemic agents by thin-layer chromatography

The use of orally administered drugs to replace insulin for the control of certain conditions of diabetes mellitus has gained popularity during the past few years. Four such compounds are listed in the recent edition of "New Drugs"[1] as being of general availability in North America. They include the three sulphonylurea derivatives, acetohexamide [1-(*p*-acetylphenylsulphonyl)-3-cyclohexylurea], chlorpropamide [1-(*p*-chlorophenylsulphonyl)-3-propylurea], and tolbutamide [1-butyl-3-(*p*-tolylsulphonyl) urea]; and the biguanide, phenformin hydrochloride [N^1-β-phenethylbiguanide hydrochloride]. The increased use of these drugs and a recent indication that combinations of hypoglycaemic agents can be more effective than the individual drugs alone in controlling diabetes[2] infer that a rapid method for their detection would be of value.

The three sulphonylurea derivatives may be classified with the general sulphonamides, the paper and thin-layer chromatography of which has been widely investigated. However, there are few references to the chromatography of the above sulphonylureas and visual detection has had limitations due to their lack of a chemical grouping suitable for colour reactions. CHAKRABARTI[3] achieved a slight difference in R_F values for the separation of chlorpropamide and tolbutamide together with carbutamide on paper using phenylhydrazine and nickel sulphate spray solutions for detection (limit 10 μg). HENTRICH[4] included tolbutamide in a series of sulphonamides separated on paper and was able to detect the hypoglycaemic agent by hydrolysis and reaction of the liberated butylamine with either ninhydrin or Folin reagent. The application of thin-layer chromatography to the separation of chlorpropamide and tolbutamide was investigated by REISCH *et al.*[5], and to the separation of tolbutamide from other sulphonamides by NEIDLEIN *et al.*[6]. SMITH *et al.*[7] separated acetohexamide from its metabolite by this technique. Apart from the use of ninhydrin in one instance[5], detection of the drugs has been by absorbance on fluorescent plates under short-wave U.V. light.

Since the chromatography of the sulphonylureas has been limited and no rapid and sensitive microchemical characterisation or specific assay is available, a thin-layer chromatographic method is described here whereby microgram quantities of the four commercially available oral hypoglycaemic agents can be conveniently separated and detected.

Experimental

Solvent systems

S_1 = Acetone–benzene–water (65:30:5),
S_2 = Acetone–butanol–water (20:50:30),
S_3 = Butanol saturated with water,
S_4 = Butanol–formamide–water (50:10:50) (upper phase used),
S_5 = Dioxane–ammonia (0.88 sp.gr.)–water (100:3:10).

Chromatographic plates

Plates (20 × 20 cm) were coated with a uniform thickness of 250 μ of silica gel G.F. (Merck) according to STAHL's method[8]. After air drying, they were activated at

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TABLE I

AVERAGE R_F × 100 VALUES FOR HYPOGLYCAEMIC AGENTS

Hypoglycaemic agent	*Solvent system*									
	S_1		S_2		S_3		S_4		S_5	
	*Range**	*Av.value*	*Range**	*Av.value*	*Range**	*Av.value*	*Range**	*Av.value*	*Range**	*Av.value*
Acetohexamide	47–57	52	66–71	67	46–53	49	48–53	50	40–45	42
Chlorpropamide	56–63	59	68–72	70	49–55	53	49–57	52	32–38	35
Phenformin HCl	—	03	49–55	52	21–27	24	29–35	32	02–03	03
Tolbutamide	72–79	75	73–79	76	62–69	65	63–70	66	31–36	33

* Experimental range representing fifty measurements.

100–110° for 30 min and left at room temperature for at least 30 min before use. The plates were used on the same day of activation.

Spray reagents

1 Ninhydrin (0.3 g) in *n*-butanol (100 ml), mixed with glacial acetic acid (3 ml)[9].

2 Vanillin (5 %) in conc. sulphuric acid.

3 Sodium nitroprusside–potassium ferricyanide spray[9]. One volume each of sodium hydroxide (10 %), sodium nitroprusside (10 %) and potassium ferricyanide (10 %) solutions was mixed with three volumes of water and the mixture left to stand for 20 min prior to use.

Method

R_F values for individual compounds were determined by the application of 5 μg quantities of an ethanolic solution of the drug to the plates and by allowing the solvent front to advance 15 cm in a previously saturated, paper lined tank. The approximate development times were: for S_1, 25 min; S_2, 110 min; S_3, 130 min; S_4, 140 min; S_5, 45 min. The method of drying and visualisation of the plates is described below. An average R_F value based on approximately fifty applications run on five plates was obtained for each compound in each solvent system. Detection limits for each compound were determined by spotting varying sample amounts with a Burroughs Wellcome "Agla" syringe.

The following scheme was found successful for identifying any of the four compounds. Two spots of 5 μg each of the material to be examined were applied to a plate and the solvent was allowed to advance 15 cm. After air evaporation of the solvent, the plate was observed under short-wave U.V. light and each hypoglycaemic agent detected by its absorbance. The plate was then heated for 10 min at 150–160° and one half of the plate (*i.e.* one spot) was sprayed with ninhydrin reagent and heated at the same temperature for a further ten minutes. Chlorpropamide and tolbutamide appeared as pink spots. The same half of the plate was sprayed further with the vanillin–sulphuric acid reagent and warmed slightly for 1–2 min. Acetohexamide was thus detected by the almost immediate appearance of a red spot. The remaining half of the plate was sprayed with the sodium nitroprusside–potassium ferricyanide reagent; a red spot appeared immediately to indicate the presence of phenformin hydrochloride.

Results and discussion

Average R_F values and their experimental range based on fifty applications of the hypoglycaemic agents in the five solvent systems are given in Table I. The limits of detection were found to be about 1 μg for the U.V. absorbance of chlorpropamide and tolbutamide, and less than 1 μg for the U.V. absorbance of acetohexamide and phenformin hydrochloride as well as for the colour reactions of all the compounds studied. The pink coloration produced by ninhydrin with chlorpropamide or tolbutamide was found to be permanent but faded on spraying with the vanillin–sulphuric acid reagent. The red colour produced by phenformin hydrochloride on treatment with the nitroprusside–ferricyanide spray was found to fade after a few minutes while that of acetohexamide with vanillin–sulphuric acid changed to a permanent orange-

brown after about 15 min, thus enhancing the identification of this drug. Phenformin hydrochloride cannot be detected by the nitroprusside–ferricyanide spray when solvent system S_4 is used. This may be attributed to the interference of residual formamide on the plate.

A mixture of all four compounds can be separated by solvent system S_1. Systems S_2 to S_4 will separate tolbutamide from phenformin hydrochloride and either acetohexamide or chlorpropamide and are intended as alternatives to system S_1. A mixture of acetohexamide and chlorpropamide will not separate in systems S_2 to S_4 and should a spot be detected which may correspond to either of these compounds alone or in combination, system S_5 will separate a mixture of the two efficiently and in conjunction with the differing colour reactions will confirm which substance is present.

This chromatographic method presents a rapid and convenient method of differentiating between the four hypoglycaemic agents and may find application in pharmaceutical or forensic work.

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Preparative polyamide layer chromatography

The rapidity of analysis, only slight broadening of the spots and easy handling in diagnostic polyamide layer chromatography[1–6] encouraged us to use preparative polyamide layer chromatography in the purification of dinitrophenyl amino acids from dinitrophenylation products. Satisfactory results were obtained and various advantages over the recrystallization method were observed in our laboratory[7]. In order to test the characteristics of the polyamide layer for preparative scale work, we selected isomeric nitroanilines for this evaluation because these compounds were brightly coloured, quite stable on handling, easily available in pure form and had large differences in R_F value.

As in previous experiments[6], we prepared the polyamide layer by spreading 15 ml of polyamide solution (20 g polycaprolactam in 100 ml of 75% formic acid) on