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DETERMINATION OF 3-(5-TETRAZOLYL) THIOXANTHONE 10,10-DIOXIDE IN HUMAN PLASMA, URINE AND FAECES

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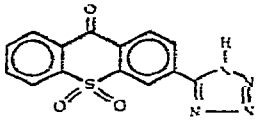
SUMMARY

A gas chromatographic method is described for assay of 3-(5-tetrazolyl) thioxanthone 10,10-dioxide (BW 59C) in human plasma, urine and faeces. After extraction into 1,2-dichloroethane from alkaline medium the compound is converted to the heptafluorobutyrate derivative which is injected into a gas chromatograph and measured using a ^{63}Ni electron capture detector. The assay produces a linear calibration curve over the range 0–30 μg/ml when the internal standard method is used. Reproducibility is good and sensitivity down to 1 ng injected on column is possible. The method has been used to investigate the pharmacokinetic properties of BW 59C in man and has been semi-automated by the use of an autosampler and dedicated computer.

INTRODUCTION

A new antiallergic compound 3-(5-tetrazolyl) thioxanthone 10,10-dioxide (BW 59C) (I) has been developed by the Wellcome Research Laboratories, the pharmacological properties of which have been described[1]. It has an action similar to sodium cromoglycate with the advantage that it is active orally. In addition to



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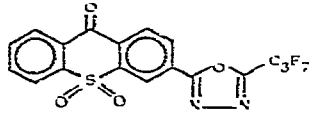

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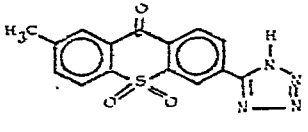

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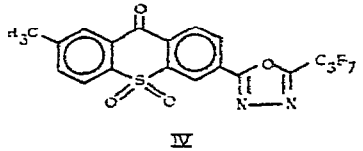

IV

in vitro activity, Haydu *et al.*[2] have shown that it inhibits asthma induced by allergen inhalation in susceptible human subjects. Because of the possible therapeutic application of the compound, it was necessary to develop a method of estimation that could be used in human plasma, urine and faeces and animal plasma and tissues. This paper describes a suitable method based on gas–liquid chromatography (GLC).

METHODS AND RESULTS

Reagents and materials

BW 59C* (Burroughs Wellcome, Dartford, Great Britain) as the free acid forms a stable monohydrate useful for preparation of standard solutions. For aqueous solutions a calculated minimum of base (preferably NaOH) must be added because solubility of BW 59C in water is low. The crystalline sodium salt, however, is hygroscopic and this must be allowed for in making standard solutions from it.

BW 374C [7-methyl-3-(5-tetrazolyl) thioxanthone 10,10-dioxide (II)] was used as the internal standard and it was handled in the same way as the free acid. Both it and BW 59C are unstable in strong base (pH > 13) but stable in acid.

The following chemicals were used: 1,2-dichloroethane (BDH, Poole, Great Britain), freshly redistilled before use; nitric acid, 10 *M* (Phoenix Chemicals, London, Great Britain); pyridine, Analar (BDH); ethanol, general purpose absolute alcohol; toluene, Analar (BDH); dimethylformamide, Analar (BDH); diethyl ether, Analar (BDH), further purified daily by passing about 200 ml down a Type "O" activated alumina 20-g column (Laporte, Widnes, Great Britain); Silyl-8 (Pierce, Rockford., Ill., U.S.A.); sodium carbonate, 0.1 *M* (BDH); heptafluorobutyric acid anhydride (HFBAA) (Pierce), of which 1-ml ampoules were used, as once opened storage is a problem because of hydrolysis by atmospheric moisture (The anhydride can be separated from the acid by fractional distillation if necessary).

Glassware

Quickfit (Q & Q) MF 24/1/6 and MF 24/1 tubes were used for the extractions and BC24/C14T tapered tubes were used for the derivatisation. Hewlett-Packard vials (2 ml) were used to store the derivatised samples before introduction into the Hewlett-Packard 7670A automatic injector unit. All glassware was thoroughly washed and silanised before use by the standard procedures. "Drifilm" (Pierce) 5% v/v in toluene was used as the silanising reagent.

Gas chromatograph

A Hewlett-Packard (Wokingham, Great Britain) 5750G gas chromatograph equipped with a standard ^{63}Ni electron capture detector was used.

Preparation of samples and standards

One-milliliter solutions containing BW 59C in plasma, urine or faeces were added to 2 ml nitric acid and 10 ml 1,2-dichloroethane in 20-ml extraction tubes. The appropriate amount of BW 374C (usually 10 μg in 10 μl) was then added as the internal standard. The tubes were tightly glass stoppered and shaken for 2 h at room

* Known as Doxantrazole.

temperature along their long axes on a Gengly rocking table (25 oscillations/min). The liquid phases were separated by centrifugation at 1000 *g* for 20 min. In order to avoid the precipitated protein and aqueous layer, care was taken when transferring 5 ml of the organic phase to the tapered reaction tubes. This was done by complete removal of the aqueous phase by aspiration and careful avoidance of the protein precipitate when pipetting. Five millilitres only of the possible 10 ml of organic phase were removed. The 1,2-dichloroethane was taken to dryness at 60° under a stream of nitrogen. The derivatisation was carried out by adding 100 μl HFBAA to the residue and after heating at 60° for 30 min, 10 μl of pyridine was slowly added followed by a further 30 min heating at 60°. During the heating time the reaction tubes were glass stoppered. Excess pyridine and fumes were removed at 70° by unstoppering the tubes and purging with nitrogen. The yellow to brown oily or solid residue was dissolved in 2 ml purified diethyl ether and 2 ml of sodium carbonate solution were added. The tubes were then shaken as above for 2 min and then 1 ml of the ether phase was transferred to a 2-ml sample vial and reduced to dryness at 60° under a stream of nitrogen. The residue was dissolved in 500 μl of 1.0% v/v DMF in toluene and then stored at 4° prior to injection of usually 1 to 2 μl into the gas chromatograph.

For some biological fluids (*e.g.*, urine) the ether extraction was omitted and the solvent extraction times shortened.

Using the above procedure more than 96% of BW 59C is converted to 3-[5-heptafluoro-*n*-propyl-2-(1,3,4-oxadiazolyl)]thioxanthone 10,10-dioxide (HF 59C) (III) as confirmed chemically. BW 374C behaves in an analogous manner to BW 59C, yielding 7-methyl-3-[5-heptafluoro-*n*-propyl-2-(1,3,4-oxadiazolyl)]thioxanthone 10,10-dioxide (HF 374C) (IV).

Gas chromatographic conditions

The 6 ft. × 4 mm I.D. glass column was hand packed with 5% OV-17 on Chromosorb W-HP (100–120 mesh) in the usual way. A ^{63}Ni electron capture detector at 315° was used. The injection port was kept at 295° and the oven at 285°. Both carrier and purge gases were 5% v/v methane in argon flowing at 20 and 45 ml/min, respectively. Under these conditions BW 59C had a retention time of 3.90 min and BW 374C one of 4.60 min and both produced good peak shapes (Fig. 1). Depending on the sensitivity required pulse intervals of 5, 15 or 50 μsec were used with a usual electrometer setting of $10^2 \times 2$. About 1 ng BW 59C injected "on column" could be detected. Peak deflection was not allowed to exceed 60% of the standing current, thus allowing the detector to operate in its linear range.

A dedicated computer (Hewlett-Packard Model 3352) was used to calculate peak areas and their ratios for the results to be described, and in addition the use of an automatic liquid sampler enabled large numbers of samples to be handled efficiently.

Calculation of results

A known mass of internal standard was added to a range of standard solutions of BW 59C and from the areas of peaks obtained after GLC separation a calibration curve was constructed with concentration of BW 59C plotted on the abscissa, and the ratio peak area BW 59C to peak area BW 374C along the ordinate. The internal standard in known mass was likewise added to the solution of BW 59C to be calcu-

Fig. 1. Typical gas chromatograms of (a) human "test" and "blank" plasma, (b) range of urine standards with from left to right 10, 20 and 30 μg/ml of BW 59C, and (c) faecal "test" and "blank" extracts.

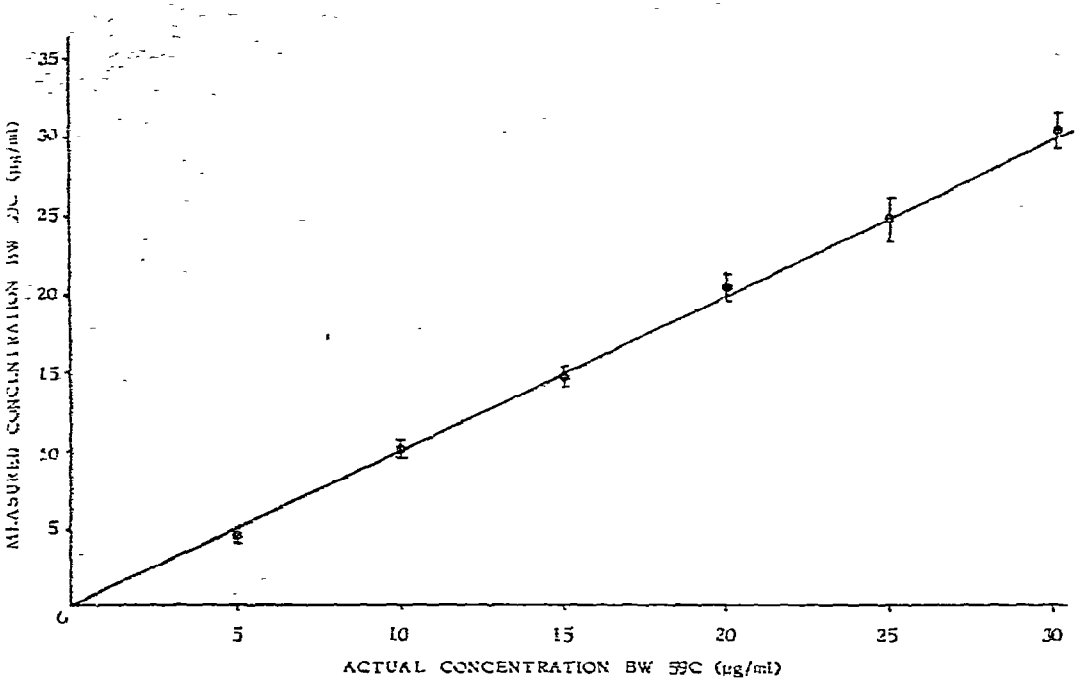

Fig. 2. Typical recovery relative to the internal standard (BW 374C) and reproducibility (± S.D.) after BW 59C was repeatedly assayed ($n = 6$) from human plasma standards.

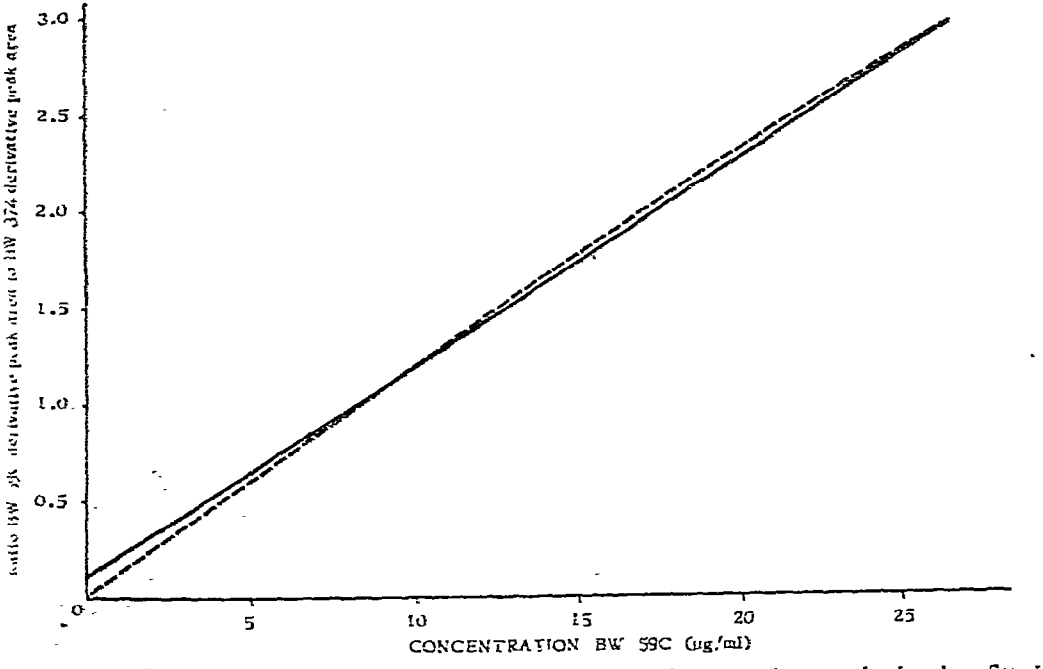

Fig. 3. Typical calibration curve for BW 59C from extracted human urine standards when fitted to a straight line (———) or curve (— — —) of the type $y = a[\tanh(bx + c) - \tanh c]$.

lated using the calibration curve. The internal standard BW 374C has closely similar physicochemical properties to BW 59C[3], and this reduces errors ascribable to variation in percentage extraction and also detector response.

Validation data

Fig. 1 shows typical chromatograms from human plasma, urine and faecal extracts. The peaks in plasma samples were well resolved. The recovery when using the automatic devices was complete relative to the drug standards in plasma, urine or faeces. A typical recovery experiment for plasma is shown in Fig. 2: urine and faeces gave similar results. A curve fitting procedure such as $y = a[\tanh(bx + c) - \tanh c]$ gave a slightly better fit if "S"-shaped standard curves were produced by the detector. However, using the described method the data points could be fitted adequately to a straight line (Fig. 3).

Table I shows replicate values for recovery of BW 59C from plasma, urine and faeces. The reproducibility of the method was good and its sensitivity high. In samples of plasma, urine and faeces from a variety of undosed patients no measurable peak appeared in the position expected of BW 59C, showing that the method was specific.

TABLE I

REPRODUCIBILITY OF THE ASSAY WHEN HUMAN PLASMA, URINE AND FAECAL SAMPLES WERE REPEATEDLY ASSAYED BY THE DESCRIBED METHOD

Sample	*Returned concentration BW 59C (μg/ml*)*						*Mean ± S.D.*
	1	*2*	*3*	*4*	*5*	*6*	
Pre-drug plasma	0	0	0	0	0	0	0
Post-drug plasma in same person	7.30	7.60	7.67	7.94	7.63	7.70	7.64 ± 0.21
Pre-drug urine	0	0	0	0	0	0	0
Post-drug urine in same person	12.10	12.11	12.22	12.08	12.20	12.10	12.13 ± 0.06
Pre-drug faeces	0	0	0	0	0	0	0
Post-drug faeces in same person	37.13	38.96	37.56	36.64	35.38	41.89	37.59 ± 2.62

* Faeces in μg/g wet weight before homogenisation.

DISCUSSION

Although the molar extinction coefficient and fluorescence of BW 59C were relatively high, problems were caused by compounds naturally present in biological fluids producing high blank values rendering methods associated with these characteristics less useful than expected. Cathode ray polarography was not used for similar reasons. A colorimetric method based on the radical anion was unsatisfactory due to dependence on light and atmospheric oxygen.

Gas chromatography proved to be adequate for the human plasma, urine and faecal extracts prepared as described. The semi-automated method handles the output

from at least two technicians (about 200 samples per week), but the data unit described will handle the output from up to sixteen chromatographs. The method has been used in several human pharmacokinetic experiments. However, in faeces peaks elute close to BW 59C and although these can be taken into account by the data system a preliminary extraction (details on request) is recommended if a manual method of peak area measurement is used.

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