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Note

Gas–liquid chromatographic determination of sulphadiazine and its major metabolite in human plasma and urine

A. BYE and G. LAND

Department of Clinical Pharmacology, The Wellcome Research Laboratories, Langley Court, Beckenham, Kent (Great Britain)

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The standard colorimetric method of Bratton and Marshall[1] for assaying sulphonamides has several disadvantages when applied to sulphadiazine (SDZ) and its major metabolite N^4-acetyl sulphadiazine (N^4-acetyl SDZ). Some of these disadvantages are overcome by high performance liquid chromatography or gas–liquid chromatography (GLC)[2,3] at the cost of convenience. A specific assay method has been developed with the appropriate sensitivity and capacity for use in pharmacokinetics and this method is described below.

MATERIALS AND METHODS

Reagents

Reagents used were: SDZ (Sigma, Kingston-upon-Thames, Great Britain); N^4-acetyl SDZ (synthesised by Dr. Hodson, Burroughs Wellcome, Beckenham, Great Britain); 9-bromophenanthrene (Koch-Light, Colnbrook, Great Britain); chloroform (BDH, Poole, Great Britain) freshly distilled before use; toluene (AnalaR grade; BDH); dichloromethane (BDH); dimethylformamide (DMF) (BDH); N,N-diethylaniline (Koch-Light); Dowtherm A (Fluka, Buchs, Switzerland); 0.18 *M* phosphoric acid (BDH); Diazald (Aldrich, Gillingham, Great Britain). Only double glass-distilled water was used.

Glassware

Soviril (Levalloix-Perret, France) 20-ml (16 mm O.D.) screw-capped test tubes were used as extraction tubes and Quickfit (Quickfit & Quartz, Corning Ltd., Stone, Great Britain) BC24/C14T stoppered tubes were used for the methylation. Hewlett-Packard (Wokingham, Great Britain) vials either 2 ml or 200 μl in volume were used to store the samples before GLC.

Apparatus

A Hewlett-Packard 5750G or Perkin-Elmer (Beaconsfield, Great Britain) F30 gas chromatograph equipped with a ^{63}Ni electron capture detector was used. Either chromatograph was linked to a Hewlett-Packard 7670A autosampler and peak integration was carried out using a Hewlett-Packard 3352 computer system.

Standard solutions

Sulphadiazine (100 mg) was dissolved in about 50 ml water containing 0.5 ml, 2 *M* sodium hydroxide and then made up to 100 ml with water. The final 1-mg/ml stock solution, with a pH of 8 was made weekly and stored at 4°. A 1-mg/ml solution of N^4-acetyl SDZ was made up in the same way.

Standard solutions of SDZ and N^4-acetyl SDZ alone or in combination were prepared by dilution of the stock solutions with water, plasma or urine, to cover the range 0–60 μg/ml. These solutions were stored at —20°.

The internal standard solution was prepared by dissolving 9-bromophenanthrene (9-BP) in dichloromethane to a final concentration of 20 μg/ml and stored at 4°. As a suitable sulphonamide-like internal standard has not yet been found 9-BP was used as an internal standard for convenience.

Column preparation

A 1.82 m × 4 mm I.D. glass column was hand packed with 5% OV-17 on Gas-Chrom Q (100–200 mesh). It was conditioned at 310° for at least 24 h, with 20 ml/min argon–methane (95:5) as carrier gas, before use.

Preparation of diazomethane in diethyl ether

This was prepared from Diazald as described[4] and stored in a flash-proof refrigerator at 2° when not in use. Suitable safety precautions were taken when handling this solution because of its toxicity[5] and explosiveness.

Preparation of samples

Extraction. A 200-μl aliquot of the standard solution or human plasma or human urine containing SDZ and/or N^4-acetyl SDZ (the major metabolite of SDZ in man) was added to a mixture of 1 ml, 0.18 *M* phosphoric acid and 0.8 ml water in 20-ml extraction tubes. Chloroform (10 ml) was added and the tubes (tightly stoppered) were shaken for 30 min at room temperature along their long axes on a gently rocking table at 25 oscillations per min. The liquid phases were separated by centrifugation at 1000 *g* for 10 min. The aqueous top layer was removed and 8 ml of the chloroform phase transferred to tapered reaction tubes. An appropriate amount (usually 400 ng in 20 μl) of 9-BP in dichloromethane was added as internal standard. To stabilise the methylation conditions as below, 40 μl of N,N-diethylaniline and 20 μl of Dowtherm A were added.

The chloroform was evaporated by a stream of nitrogen at 60°, leaving a yellowish oil.

Derivatisation by methylation. This process was carried out in a well ventilated fume cupboard with the technician wearing protective clothing. The oil in each tube obtained from the extraction was mixed well with 200 μl of freshly made diazomethane and then left for 10 min. The excess diazomethane and ether were removed by placing the tubes under a stream of nitrogen in a fume cupboard at room temperature for 40 min.

The resulting oily residue was then dissolved in a suitable amount of 1% DMF in toluene and transferred to the appropriate vial and stored at 4° prior to injection, using the Hewlett-Packard autosampler.

Gas chromatography

For the Hewlett-Packard 5750G a fully conditioned column was connected to the ^{63}Ni electron capture detector and injector. The injection port temperature was 295°, the oven 285° and the detector 330° with a pulse interval of 5 or 15 pulses per sec. Both carrier and purge gases were argon–methane (95:5) flowing at 46 and 20 ml/min, respectively. The Perkin-Elmer F30 was set up to give identical analytical conditions.

The sample was introduced into the gas chromatograph by the autosampler and a dedicated computer was used in conjunction with a 7123B recorder (Hewlett-Packard) to calculate peak areas and their ratios. An automatic liquid sampler enabled efficient handling of up to 36 samples per run.

RESULTS AND DISCUSSION

Under the conditions stipulated, methylated SDZ had a retention time of 4.92 min, methylated N^4-acetyl SDZ 10.67 min and the internal standard 1.25 min. The peaks were well resolved (Fig. 1). Less than 1 ng of SDZ or its metabolite could be seen when injected onto the column.

The data presented in Fig. 1 were calculated by adding a known mass of the internal standard to a range of standard solutions of sulphadiazine and/or N^4-acetyl sulphadiazine. A calibration curve was constructed by plotting the concentration of

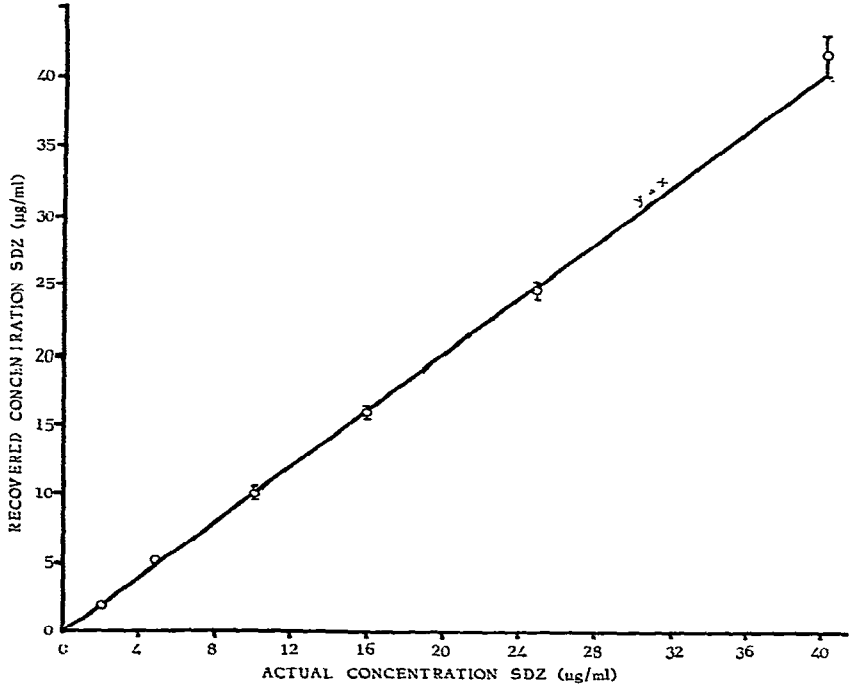

Fig. 1. The recovery relative to the internal standard when SDZ was repeatedly assayed ($n = 6$) from human plasma standards. The line $y = x$ does not differ significantly ($p < 0.025$) from the best fit to the data points.

sulphonamide against the ratio of the areas of the peaks for methylated sulphonamide and 9-BP. The internal standard in known mass was likewise added to the unknown sample (urine or plasma), extracted and the amount of SDZ and/or N^4-acetyl SDZ calculated from the calibration curve. The results (Fig. 1) show that recovery of SDZ added to plasma is complete relative to the drug standards. An equivalent result was obtained with N^4-acetyl SDZ recovery from plasma. Recovery from urine gave similar results. The calibration curves were adequately described by a straight line using the detection system stated. Hence no curve fitting procedures were needed.

Table I shows replicate values for recovery of SDZ and N^4-acetyl SDZ from plasma and urine. The reproducibility of the method was good and its sensitivity high. In samples of plasma and urine from undosed patients no measurable peaks occurred in the positions expected of SDZ and N^4-acetyl SDZ showing that the method was specific (Fig. 2).

TABLE I

THE PRECISION OF THE ASSAY WHEN SDZ AND N^4-ACETYL SDZ WERE ANALYSED IN UNKNOWN HUMAN PLASMA AND URINE

Sample	*Concentration (μg/ml)*						*Mean*	*Standard Deviation*
	1	*2*	*3*	*4*	*5*	*6*		
Sulphadiazine								
Plasma 1	29.85	32.40	31.10	28.35	27.25	27.20	29.36	± 2.13
2	9.68	10.57	10.52	9.78	9.51	10.04	10.02	± 0.44
Urine 1	11.58	11.72	10.69	12.64	11.77	11.23	11.61	± 0.65
N^4-Acetyl sulphadiazine								
Plasma 1	10.66	11.00	11.56	10.27	9.85	9.48	10.47	± 0.76
2	1.93	2.28	2.28	2.08	2.05	1.97	2.10	± 0.15
Urine 1	4.32	4.45	4.26	4.73	4.63	4.23	4.44	± 0.21

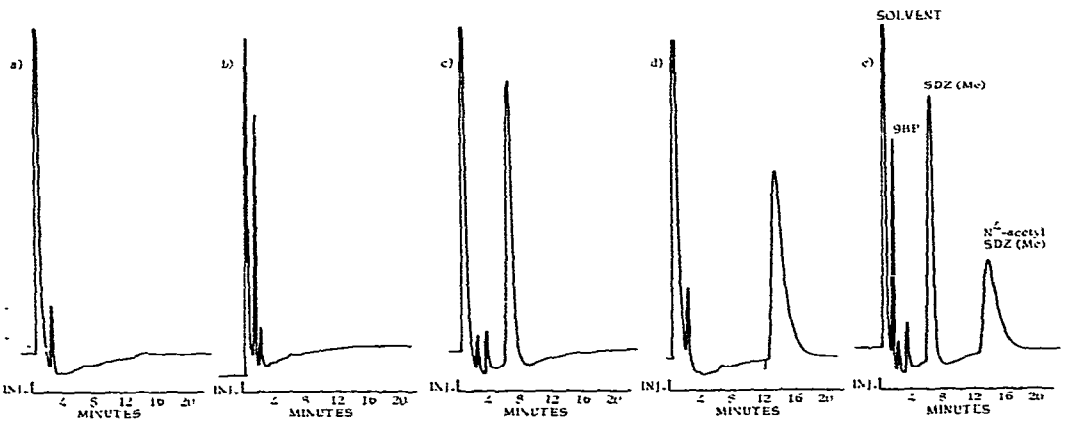

Fig. 2. The chromatograms produced from human plasma when (a) "blank", (b) internal standard only, (c) SDZ only, (d) N^4-acetyl SDZ only and (e) SDZ (10 μg/ml), N^4-acetyl SDZ (10 μg/ml) and internal standard were analysed by the described procedure.

The described method satisfactorily meets the needs of human pharmacokinetic and bioavailability studies. About 70 samples per working day can be analysed using the described apparatus and two technicians.

Although many methods are available for sulphonamide measurement in human plasma and urine they are usually based on the colorimetric method of Bratton and Marshall[1]. Appreciable hydrolysis of N^4-acetyl SDZ occurs before the boiling step in this procedure which results in high representation of unchanged SDZ before hydrolysis. The total SDZ levels would not be expected to be affected. Apparently high levels of unhydrolysed SDZ appear in the urine of some subjects before they took SDZ. This spurious substance disappears on boiling the urine. The use of the colorimetric technique has these two problems which can result in erroneously low levels and apparently negative quantities of metabolites. The first problem seems to be marked with SDZ but presumably could be overcome if neutral conditions were used. However the second problem would produce errors in all sulphonamide assays based on the Bratton–Marshall procedure. In most cases where high unchanged sulphonamide levels are encountered ($>40\ \mu g/ml$) the urine "blank" would add little. However at low concentrations (less than $20\ \mu g/ml$) the error caused by the urine blank would be large and in fact the colorimetric procedure falters at this level for this reason. As the SDZ levels after conventional dosing are in this $20\ \mu g/ml$ region alternative methodology is preferable to further modifications of the colorimetric procedure.

The GLC method described has specificity since other sulphonamides such as sulphapyrazine, sulphafurazole, sulphanilamide and sulphamethoxazole do not interfere; and sensitivity since levels as low as 1 ng injected on-column could be detected. No hydrolysis is needed for the simultaneous assay of N^4-acetyl sulphadiazine. The only disadvantage is that extraction and derivatisation are needed.

The described method was semi-automated and can be used routinely to measure sulphadiazine and its major metabolite in human plasma and urine.

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