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Note

Rapid gas chromatographic determination of ifosfamide in biological fluids

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Ifosfamide [IF, 3-(2-chloroethyl)-2-(2-chloroethylamino)tetrahydro-1,3,2-oxazophosphorine 2-oxide] is a structural analogue of cyclophosphamide in which one functional chloroethyl group is transferred from the extracyclic to the endocyclic nitrogen. IF has efficacy in testicular tumours including teratomas, osteosarcoma, lymphoma, pancreatic carcinoma and small cell bronchial carcinoma [1—4]. Like cyclophosphamide, IF requires metabolic activation to alkylating metabolites which have anti-mitotic actions. These latter compounds, only some of which are presently known, cannot at the moment be satisfactorily or easily estimated in biological fluids. Therefore pharmacokinetic analysis of the disposition of IF and its relationship to efficacy and dosage regimes must largely rest upon the estimation of IF and alkylating activity in plasma.

Methods for IF estimation by gas chromatography (GC) have previously been published. The earliest published method was for underivatised IF and separated IF from other compounds on a 3.8% W-982 column followed by flame-ionisation detection. The technique required, however, 2 ml plasma and a complicated extraction procedure to yield a sensitivity of only 1 μg/ml [5]. Greater sensitivity was achieved by conversion to the trifluoroacetyl (TFA) derivative and chromatography using electron-capture (ECD) or nitrogen—phosphorus detection [6—8] which allowed considerably better selectivity and sensitivity. The estimation of underivatised oxazophosphorines is complicated by the occurrence of intramolecular alkylation. This can be prevented by derivatisation [6—8] or by careful selection of assay conditions. Such conditions have been determined for cyclophosphamide and, by implication, for IF [9]. Recently an ECD—GC method for IF determination has been described [10] which requires derivatisation using heptafluorobutyric

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anhydride. The present report describes a practical assay for underivatised IF in biological fluids and compares it with a more complex assay which requires prior trifluoroacetylation of IF. The use of the underivatised technique for IF estimation is exemplified by a study of IF disposition in a dog.

EXPERIMENTAL

Chemicals

IF was a gift from WB Pharmaceuticals (Bracknell, U.K.); trophosphamide was donated by Prof. Dr. N. Brock, Asta-Werke (Bielefeld, F.R.G.). Other chemicals were purchased from BDH (Poole, U.K.).

Instrumentation

The chromatograph was a Pye 104 model fitted with a glass column (0.9 m × 2 mm I.D.) packed with 5% SE 30 on 80—100 mesh Chromosorb W (acid-washed, DMCS-treated). It was operated at 200°C with a nitrogen (carrier) flow-rate of 40 ml/min. The nitrogen detector was maintained at 350°C. The flow-rates were 65 ml/min and 635 ml/min for hydrogen and air, respectively.

Analytical methods

For direct assay of ifosfamide, the internal standard (5 μg trofosfamide) and 0.2 ml of 0.1 *M* sodium hydroxide were added to 1 ml of plasma containing IF and briefly mixed. This mixture was extracted with 3 ml ethyl acetate by mixing for 1 min on a vibration mixer (Whirlimixer; Luckhams, Burgess Hill, U.K.). After centrifugation for 10 min at 1000 *g*, 1 ml of the supernatant was transferred to a clean dry tapered glass tube (15 ml) and evaporated to dryness in a gentle stream of air in a 50°C waterbath. The dried residue was redissolved in 100 μl ethyl acetate and 2 μl of this were injected into the gas chromatograph. The volumes given above are suitable for clinical samples; adjustment of volumes will allow greater sensitivity for other circumstances.

Assay of TFA-derivatised IF was by a previously published method [6].

The 4-(4-nitrobenzypyridine) pyridine (NBP) alkylating activity was measured spectrophotometrically [7].

RESULTS

Typical chromatograms from the assay of underivatised IF are shown in Fig. 1. The retention times were 0.6 min for IF and 1.3 min for the internal standard. Identification was made by retention time in comparison with standards and peak superimposition. No interference was noted from endogenous plasma constituents. Standard solutions of IF in blank human plasma gave excellent linearity with respect to ratio of peak heights of IF:internal standard over a range of plasma concentrations from 1 to 50 μl/ml ($r = 0.999$; $n = 22$; $P < 0.001$). The relative standard deviation of between-assay replicates over this range of concentrations varied from 0.06 to 0.11. The minimum detection level (signal-to-noise ratio 2:1) was 100 ng/ml for plasma (Fig. 1).

The accuracy in assay of known IF concentrations in spiked plasma samples

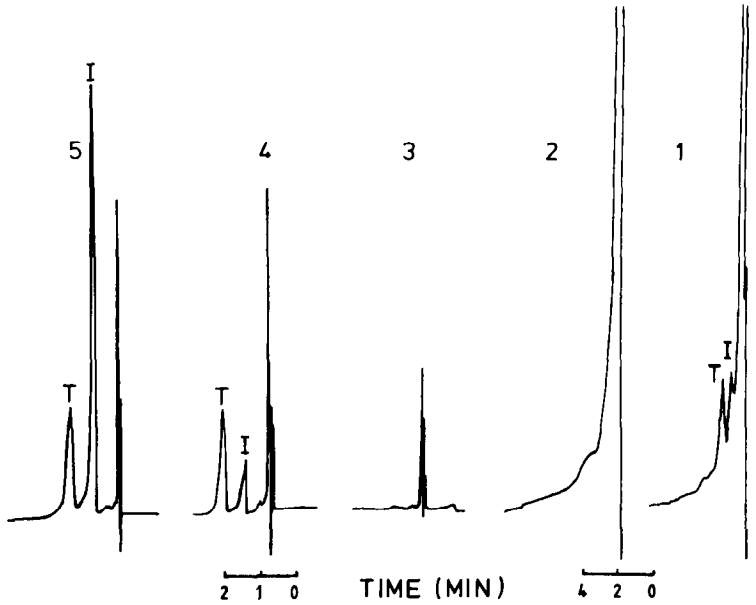

Fig. 1. Representative chromatograms from plasma. Injection artifact is first peak on right and all chromatograms are read from right to left. Chromatograms: (1) blank plasma containing 100 ng/ml ifosfamide; (2) the corresponding blank plasma; (3) blank plasma recorded at the attenuation appropriate to demonstrate the peaks due to 2 μg/ml ifosfamide (4); and 10 μg/ml ifosfamide (5). Peaks: T, trophosphamide (internal standard); and I, ifosfamide.

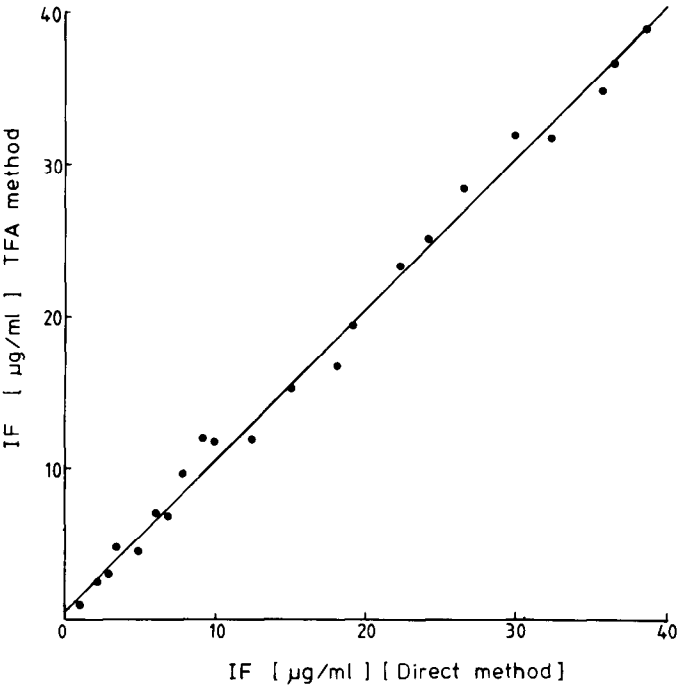

Fig. 2. Relationship between 22 estimates for IF concentrations assayed from the same sample by the direct and TFA-derivatisation methods.

was excellent. The correlation coefficient between known and estimated IF concentrations was 0.999 with a slope of 0.989 (n = 22). The intraclass correlation coefficient R_i was 0.997 indicating similarity of slope and intercept of the regression [11].

To compare the direct assay of IF with its assay as the trifluoroacetylated derivative, 22 plasma samples containing IF concentrations between 1 and 40 μg/ml were assayed by both techniques. Fig. 2 shows the results of this study. The least-squares regression line shown in Fig. 2 is derived from the equation [TFA method result] = 0.988 [direct method result] + 0.61. The 95% confidence limits of the intercept are 0.38—0.84 and of the slope 0.949—1.028. It will be noted that the latter interval spans unity which would be the slope for perfect parallelism. For this regression, r = 0.996 ($P < 0.001$). The intraclass correlation coefficient R_i tests for concordance and was 0.984. A residual plot indicated no significant bias and this was confirmed by analysis of variance.

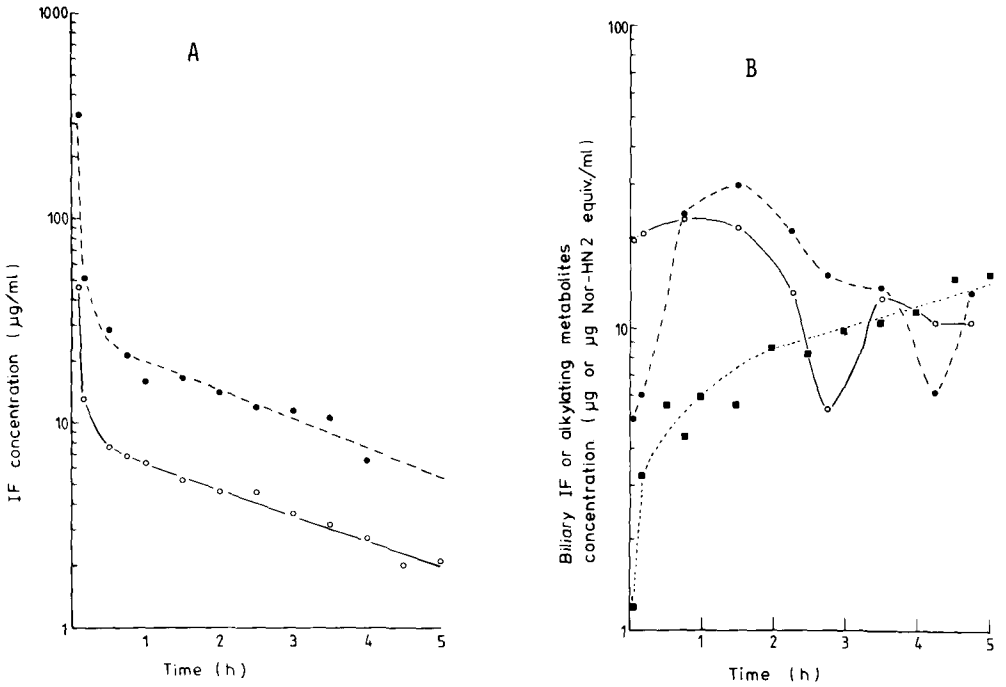

Fig. 3. (A) Plasma concentration—time profiles of unchanged IF in the femoral (•) and hepatic portal (○) veins of a dog following intravenous injection of 20 mg/kg IF. (B) Total NBP alkylating activity in the same dog (expressed as μg nornitrogen mustard equivalents per ml) determined in femoral vein (▪) and bile (•) compared with elimination of unchanged IF in bile (○).

Fig. 3 shows the plasma concentrations of unchanged IF in the femoral artery, hepatic portal vein and bile of a male greyhound (27.5 kg) given IF 20 mg/kg as an intravenous bolus following cannulation under general anaesthesia of the appropriate vessels. The half-life in the β-phase of IF in both femoral arterial and hepatic portal venous plasma was 2.3 h, the apparent volume of distribution in the β-phase of the central compartment (V_β) 1.0 l/kg, and the systemic clearance 0.3 ml/kg/h. IF elimination in bile showed a discontinuous pattern and this was mirrored by elimination of NBP-alkylating

activity. There was only moderate correlation between biliary and femoral artery IF concentrations ($r = 0.78$; $n = 6$).

IF may also be estimated in urine from patients undergoing treatment for sarcomas using this underivatised method [12].

DISCUSSION

The assay for estimation of underivatised IF was found to be as accurate as that involving the more complex requirements of derivatisation with trifluoroacetic acid. The extraction time is rapid and chromatography on a relatively narrow-bore column gives a short retention time so that some 50 samples can easily be assayed in a working day. A previous report suggested decompositon of underivatised IF on an SE 30 column [8]; this was not noted in the present study. Under the present assay conditions, cyclophosphamide shows formation of double peaks and has a brief retention time which made it unsuitable as an internal standard. An assay for underivatised cyclophosphamide has previously been published [9].

In our hands this rapid assay has proved suitable for pharmacokinetic studies of IF in animals and man using samples of saliva, plasma, bile and urine. The assay is suitable for pharmacokinetic studies as illustrated by a study of IF kinetics in bile and plasma following intravenous administration to a dog. The elimination of IF and its NBP alkylating metabolites in bile has not been previously reported but is consonant with analogous observations on cyclophosphamide made in man [13]. This route of elimination may explain the finding of IF in faecal samples from patients receiving IF by the intravenous route [14]. Further studies will be required to elucidate the kinetics of this in the bile but a quantitative study of cyclophosphamide in man [11] suggests by analogy that the fraction of the drug dose eliminated by this route is likely to be small.

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