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# COMBINED THIN-LAYER CHROMATOGRAPHY–PHOTOGRAPHY–DENSITOMETRY FOR THE QUANTITATION OF CYCLOPHOSPHAMIDE AND ITS FOUR PRINCIPAL URINARY METABOLITES

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## SUMMARY

A novel method for the quantitative determination of the anti-cancer drug cyclophosphamide and its principal urinary metabolites 4-oxocyclophosphamide, carboxyphosphamide, phosphoramide mustard and bis(2-chloroethyl)amine has been devised. The assay combines adsorption of drug-related material onto Amberlite XAD-2 and thin-layer chromatography with spot visualization using 4-(4-nitrobenzyl)pyridine, rapid photography and densitometry. The intra-assay coefficient of variation for each compound was <6%. The limit of detection of the assay was 1 $\mu g\ ml^{-1}$ for cyclophosphamide, phosphoramide mustard and bis(2-chloroethyl)amine and 0.5 $\mu g\ ml^{-1}$ for 4-oxocyclophosphamide and carboxyphosphamide. The method was validated for cyclophosphamide and 4-oxocyclophosphamide using gas chromatography. It is concluded that the method provides the first means of determining the full metabolic spectrum for cyclophosphamide in patients without recourse to the administration of radioisotopically labelled drug.

## INTRODUCTION

Cyclophosphamide (CP) is a DNA-alkylating agent first synthesized in 1958 [1] which has found widespread use in the treatment of malignancy [2]. In mammalian species including man CP is converted by the hepatic cytochrome P-450 mono-oxygenases to the transient chemically reactive metabolite 4-hydroxycyclophosphamide (4HCP), [3,4]. This intermediate is in equilibrium with what is often referred to as its tautomer aldophosphamide (AP), which can either be detoxicated by aldehyde dehydrogenase (ALDH) giving carboxyphosphamide (CX) or alternatively can undergo $\beta$-elimination of acrolein (propenal) to yield

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the ultimate alkylating entity phosphoramide mustard (PM), which in turn is cleaved to the highly reactive species bis(2-chloroethyl)amine ("nornitrogen mustard", NNM) [5–8]. Finally, a second detoxication pathway, presumably arising from 4HCP by dehydrogenation or from CX by ring-closure, is the production of 4-oxocyclophosphamide (4-ketocyclophosphamide; KP). The metabolic scheme showing the bifurcation between the detoxication and activation options is given in Fig. 1. The literature concerning CP metabolism is vast and the reader is directed to a recent review by Sladek [9].

Since the metabolism of CP, perhaps more than for any other drug, holds the key to both drug efficacy and toxicity in patients, it would be desirable to measure not only CP but also to have a quantitative appreciation of the relative concen-

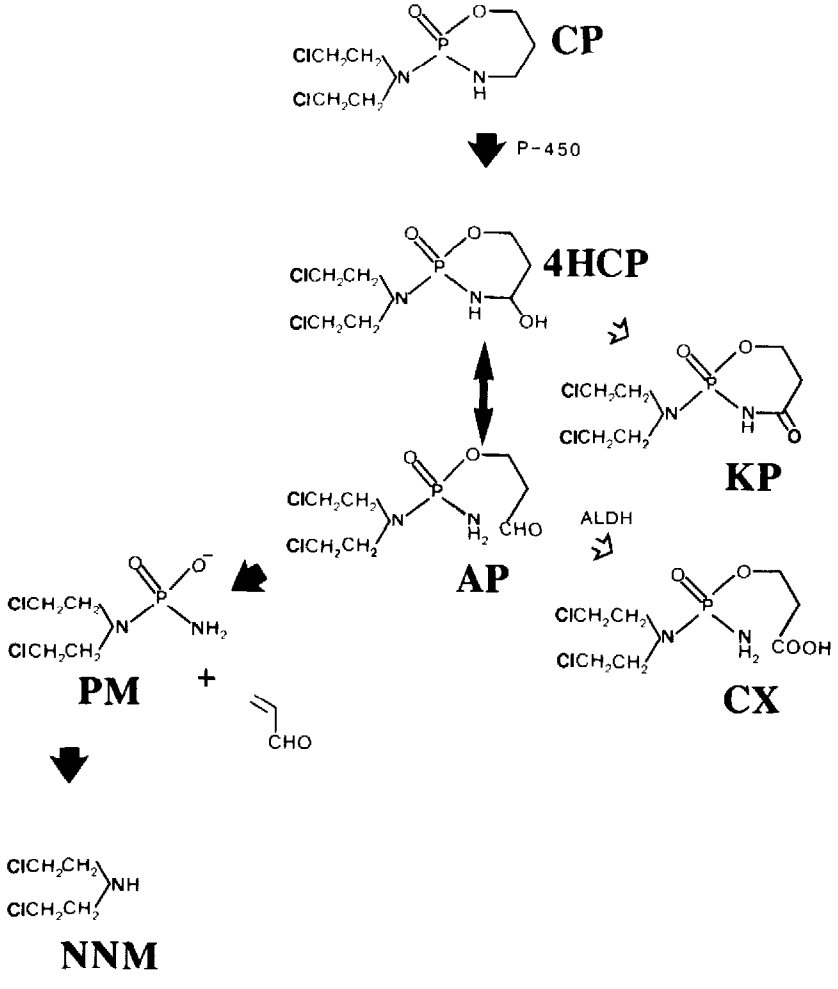


Fig. 1. Scheme for the metabolism of cyclophosphamide (CP) in man showing the chemical structures of the authentic metabolites used. Bold arrows denote activation (cytotoxic) pathways, open arrows denote detoxication reactions. See text for abbreviations.

trations of the metabolites KP, CX, PM and NNM. As can be seen from Fig. 1 this requires analysis of chemical types as diverse as the acidic PM, the basic NNM, the zwitterionic CX and the near neutral KP and CP. It is little wonder that the chromatographic methods employed to date are limited in the number of drug-related compounds which they can separate and quantitate [10–14]. As a result, investigators have traditionally chosen to measure "total alkylating activity" using the low specificity Epstein colorimetric assay which employs alkylation of 4-(4-nitrobenzyl)pyridine (NBP) [15]. In order to separate, visualize and quantitate the major metabolites of CP, we have combined a thin-layer chromatographic separation with NBP visualization, rapid photography of the labile blue spots and densitometry, hereafter referred to as combined thin-layer chromatography–photography–densitometry (TLC–PD).

## EXPERIMENTAL

### *Materials*

Chemical structures of the authentic compounds analysed are given in Fig. 1. Chemical nomenclature used is that cited in Chemical Abstracts together with the widely used trivial nomenclature of the metabolites. The following substances (CAS registry number in square brackets) authenticated by both $^1H$ nuclear magnetic resonance and infrared spectroscopy at Asta-Werke (Bielefeld, F.R.G.) were the gift of Boehringer Ingelheim (Bracknell, U.K.).

N,N-Bis(2-chloroethyl)tetrahydro-2H-1,3,2-oxazaphosphorin-2-amine 2-oxide [50-18-0] (cyclophosphamide, CP; $C_7H_{15}Cl_2N_2O_2P \cdot H_2O$ requires: C, 30.11; H, 6.09; N, 10.04%; found: C, 30.83; H, 5.96; N, 10.32%).

3-[Amino[bis(2-chloroethyl)amino]phosphinyl]oxylpropanoic acid [22788-18-7] (carboxyphosphamide, CX; $C_7H_{15}Cl_2N_2O_4P$ requires: C, 28.67; H, 5.12; N, 9.56%; found: C, 28,83; H, 5.08; N, 9.41%).

2-[Bis(2-chloroethyl)amino]tetrahydro-4H-1,3,2-oxazaphosphorin-4-one 2-oxide [27046-19-1] (4-ketocyclophosphamide, KP; $C_7H_{13}Cl_2N_2O_3P$ requires: C, 30.55; H, 4.73; N, 10.18%; found: C, 31.53; H, 4.86; N, 9.95%).

N,N-Bis(2-chloroethyl)phosphorodiamidic acid [10159-53-2] (phosphoramide mustard, PM; $C_4H_{11}Cl_2N_2O_2P \cdot H_2O$ requires: C, 20.08; H, 5.44; N, 11.72%; found: C, 20.14; H, 4.40; N, 11.31%).

2-Chloro-N-(2-chloroethyl)ethanamine [334-22-5] (nornitrogen mustard, bis(2-chloroethyl)amine, NNM; $C_4H_{11}Cl_2N \cdot HCl$ requires: C, 26.89; H, 5.60; N, 7.84%; found: C, 27.55; H, 5.57; N, 7.62%).

N,3-Bis(2-chloroethyl)tetrahydro-2H-1,3,2-oxazaphosphorin-2-amine 2-oxide [3778-73-2] (ifosfamide, IF, internal standard for the GC assay; $C_7H_{15}Cl_2N_2O_2P$ requires: C, 32.18; H, 5.75; N, 10.73%; found: C, 32.59; H, 5.77; N, 10.89%).

As can be seen, the elemental analysis on the above chemically reactive compounds was not always ideal, indicative of a small degree of decomposition. In our hands, however, they were each >98% pure by TLC analysis (vide infra) and were stored desiccated at −20°C.

Inorganic reagents were of AnalaR grade and organic solvents were HPLC grade.

4-(4-Nitrobenzyl)pyridine (NBP) was obtained from Sigma (Poole, U.K.) and trifluoroacetic anhydride was obtained from Pierce & Warriner (Chester, U.K.).

*Preparation of samples for TLC*

Urines from cancer patients receiving intravenous CP or blank human urine spiked with authentic standards (1–250 $\mu g\ ml^{-1}$) were treated as follows: urine (1.00 ml) was applied to a small column (8 cm×0.6 cm) of Amberlite XAD-2 non-ionic polymeric adsorbent (Aldrich, Gillingham, U.K.) in a glass Pasteur pipette and allowed to elute under gravity. These small XAD-2 columns had been pre-washed with successive volumes of acetone (3 ml), methanol (3 ml) and water (3 ml) to remove monomers and inorganic salts. After elution of the urine, the columns were washed with glass-distilled water (3 ml) and the columns sucked dry with a water pump, after which CP and its metabolites were eluted with methanol (5 ml) and reduced to dryness in vacuo at 40°C using a Savant Speedvac evaporating centrifuge (V.A. Howe and Company, Croydon, U.K.).

*TLC procedures*

Dry residues from the above procedure were reconstituted in methanol (30 $\mu l$) which was applied to TLC plates [precoated high-performance thin-layer chromatographic (HPTLC) aluminium-backed silica gel 60 $F_{254}$, 20×20 cm; E. Merck, Darmstadt, F.R.G.] which had been pre-eluted with methanol and dried at 150°C for 2×10 min. Chromatography was carried out in glass TLC tanks, saturated with solvent and using chloroform–ethanol–glacial acetic acid (100:20:1, v/v/v) as mobile phase and run to a height of at least 18 cm. Plates were removed, dried with cold air and sprayed for at least 15 s with 5% NBP in acetone–0.2 *M* acetate buffer pH 4.6 (8:2, v/v), dried with cold air and resprayed for 15 s. Plates were dried in air and then heated in an oven at 130–150°C for 5–15 min. After cooling, plates were dipped in 3% methanolic potassium hydroxide whereupon typical blue spots appeared, formed by the alkylation of NBP by 2-chloroethylamine derivatives, followed by proton abstraction to yield the chromophore [15]. These blue spots are unstable and therefore for purposes of quantitation the plates were photographed within 10 s of the final alkali step (vide infra).

*Photographic procedure*

With the wet TLC plate 30 cm below the film plane of a 5×4 inch bellows camera, two tungsten halogen 1.275-kW lights illuminated the plate at a 45° angle from either side and the plate was photographed using a 150-mm lens, stopped down to f11 with a one-eighth exposure onto Ilford FP4 125 ASA medium grain and contrast black and white panchromatic film. To ensure uniform exposure, each plate photographed included a Kodak standard grey scale. Once exposed, the negative was developed in complete darkness for 2 min at 20°C with constant agitation in Ilford Contrast FF, diluted five-fold. After development, the negative was rinsed quickly in an acetic acid stop bath and then fixed, washed and dried. The dry negative was enlarged using a 150-mm lens, stopped down to f22, to exactly ×1 magnification of the original TLC plate for approx. 7 s. Kentmere resin-coated glossy paper (grade 2) was used and was processed for 1 min

at 20°C with constant agitation in Ilford Ilfospeed, diluted ten-fold. The original grey scale was then compared with that on the photographic print and the negative reprinted if necessary to ensure a true photographic facsimile of the TLC plate had been obtained.

*Densitometric analysis*

For the purpose of densitometric quantitation of TLC spots, photographs were mounted on uncoated glass TLC plates and placed on the stage of a dual-wavelength TLC scanner (Shimadzu Model CS-930; Dyson Instruments, Houghton-le-Spring, Tyne-and-Wear, U.K.) fitted with a Shimadzu DR-2 data recorder. Chromatographic lanes on the photographs were scanned with a wavelength of 370–600 nm in the reflection mode with background subtraction and using a light spot of $1 \times 2$ mm. Care was taken to ensure that the zig-zag excursion of the light spot encompassed each complete chromatographic spot. Chromatograms were plotted at low sensitivity and peak areas calculated by the data recorder. Under these conditions the $R_F$ values for CP and its metabolites were: CP, 0.61; CX, 0.26; KP, 0.67; PM, 0.02; and NNM, 0.40. The method was calibrated using blank urines containing known amounts of authentic standards (1–250 $\mu$g ml$^{-1}$) taken through the above procedures. Accordingly, the concentration of CP and its principal metabolites in patient urines could be determined by the comparison of peak areas of unknowns with calibration curves.

*Validation of the TLC assay by gas chromatography (GC)*

The following GC method was used for the determination of CP and KP in samples also analysed by TLC; using a range of pH, it was found that CP and KP gave maximum efficiencies of extraction from urine into ethyl acetate at pH 14 and 4.6, respectively. Therefore, to urine (100 $\mu$l) was added IF (20 $\mu$l of a 50-$\mu$g ml$^{-1}$ methanolic solution) as internal standard, 1 *M* sodium hydroxide (1 ml) and ethyl acetate (3 ml). Mixtures were mechanically shaken for 15 min in the horizontal, centrifuged at 200 *g* for 5 min and ethyl acetate layers (2 ml) aspirated and evaporated in vacuo using the evaporating centrifuge as previously described. To the dry residue was added trifluoroacetic anhydride (100 $\mu$l) and ethyl acetate (100 $\mu$l) and derivatization achieved at 70°C for 20 min in 4-ml screw-topped PTFE-lined septum vials. After cooling, samples were evaporated to dryness as before and the residue reconstituted in dry ethyl acetate (50 $\mu$l), aliquots (1 $\mu$l) of which were subjected to GC analysis.

For analysis of KP content, to urine (1.00 ml) was added IF (50 $\mu$l of 50 $\mu$g ml$^{-1}$ methanolic solution) as internal standard, 0.2 *M* sodium acetate buffer, pH 4.6 (1 ml) and ethyl acetate (3 ml) and the procedure followed as described above for CP extraction and derivatization.

A PU4500 gas chromatograph (Philips, Cambridge, U.K.), fitted with an alkaline flame ionization detector and interfaced with both a PU4700 autoinjector and PU4810 computing integrator, was used for the determination of CP and KP. Oven temperatures were 240 and 250°C for CP and KP analysis, respectively. Nitrogen carrier gas was used at flow-rates of 35 and 30 ml min$^{-1}$ for CP and KP analyses, respectively. In both cases, injector and detector temperatures were

300°C. For both assays an OV-17 packed column (3% on Chromosorb W HP; Jones Chromatography, Llanbradach, U.K.; 2.4 m×4 mm i.d.) was used. The assays were calibrated against known standards in urine (1–100 $\mu$g ml$^{-1}$ for both CP and KP) using a peak-area ratio method.

## RESULTS

### *Chromatographic method*

Under the conditions described, all authentic CP metabolites separated well. Photography of the TLC plates gave a permanent record of the blue spots which could then be scanned using a densitometer for the purposes of quantitation. Fig. 2 shows such a TLC–PD scan of human urine spiked with CP metabolites. Calibration curves constructed up to 250 $\mu$g ml$^{-1}$ were linear. Table I gives the correlation coefficients and slopes of the calibration curves, together with the recoveries for each metabolite in the XAD-2 extraction procedure. Whilst the losses during extraction were variable, the correlations for each determination were good (0.984–0.998). Intra-assay coefficient of variation was <6% for each

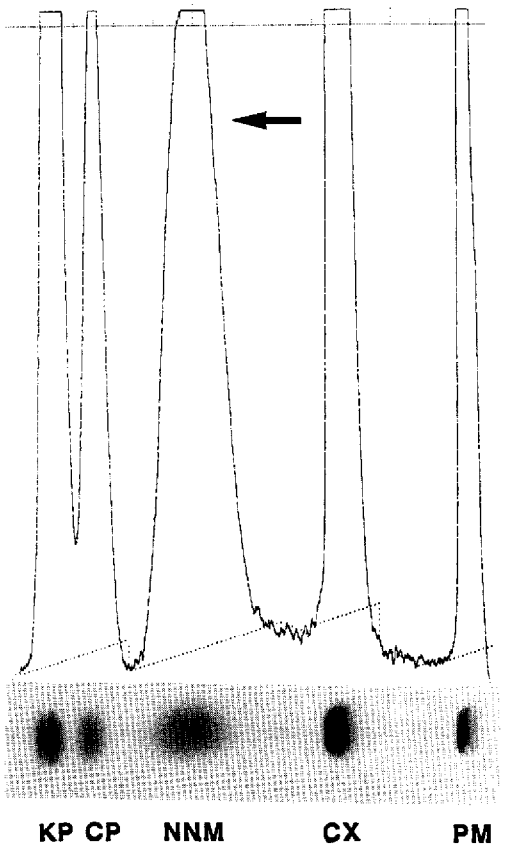


Fig. 2. TLC–PD scan (above) of authentic standards in blank human urine. Photograph of a TLC plate is shown (below). Arrow denotes direction of elution. See text for abbreviations.

TABLE I

EXTRACTION RECOVERIES, $R_F$ VALUES AND CALIBRATION CHARACTERISTICS FOR CYCLOPHOSPHAMIDE AND ITS METABOLITES IN HUMAN URINE

| Metabolite | Recovery (%) | $R_F$ | $r$ | Slope ($\times 10^{-3}$) |
|---|---|---|---|---|
| KP | 69.0 | 0.67 | 0.992 | 26.3 |
| CP | 72.3 | 0.61 | 0.984 | 15.4 |
| NNM | 60.6 | 0.40 | 0.996 | 17.5 |
| CX | 84.1 | 0.26 | 0.998 | 24.8 |
| PM | 57.0 | 0.02 | 0.991 | 9.1 |

compound and when one photograph was scanned ten times, the coefficient of variation never exceeded 2.2% for any spot. Clearly the assay is more sensitive for detecting some metabolites than others. Even when the slopes of the calibration lines (Table I) are corrected for recovery and molecular mass ($M_r$) there is still a three-fold variation in slope with the following rank order: KP > CX > CP > NNM > PM. There is therefore no relationship between the rate of alkylation of NBP on the TLC plate and biological alkylation since NNM and PM are recognized as the most potent alkylators in vivo [9]. Blank patient urines put through identical procedures demonstrated the absence of interfering endogenous compounds. However, it is envisaged that other NBP reactive alkylating nitrogen mustards such as ifosfamide and chlorambucil would, if administered concomitantly with CP, interfere with the assay. Such combinations find little clinical use. The limit of detection of the assay (signal-to-noise ratio > 2) was 1.0 $\mu$g ml$^{-1}$ for CP, PM and NNM and 0.5 $\mu$g ml$^{-1}$ for KP and CX, which is comparable to the GC assay for CP and KP described hereafter.

*Validation of TLC–PD by GC*

Because of the novelty of the TLC–PD approach to the measurement of CP metabolites, particularly the inclusion of the rapid photographic step, we wished to validate the new procedure using a well-established method. GC with nitrogen-selective detection of the trifluoroacetylated derivatives of CP and KP was chosen for this purpose. Fig. 3 shows the GC traces for the respective CP and KP determinations. The retention times of CP and IF (internal standard) were 2.7 and 3.6 min, respectively and, in the second assay, the retention times for IF and KP were 3.4 and 6.7 min, respectively. Calibration curves for both assays were linear over the range 1–100 $\mu$g ml$^{-1}$. The inter- and intra-assay coefficients of variation were < 4.9% and < 3.0%, respectively for both assays.

Twenty-one urine samples from patients receiving intravenous CP were analysed for both CP and KP by both TLC–PD and GC as described. No significant differences were observed between the concentration of CP ($t = 0.710$; $p = 0.486$) and KP ($t = 0.808$; $p = 0.428$) as judged by a paired Student's $t$-test and the values correlated well for both CP ($r = 0.949$; $p < 0.0001$) and KP ($r = 0.953$; $p < 0.0001$).

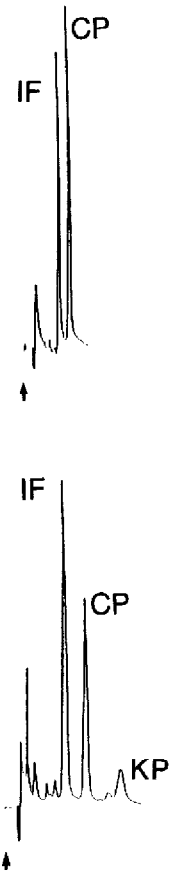


Fig. 3. GC with nitrogen-selective detection of trifluoroacetyl derivatives for the determination of CP (above) and KP (below). Arrows denote injection. See text for abbreviations.

These findings were taken as validating the precision and accuracy of the TLC–PD method.

*Pattern of CP metabolites in human urine*

The TLC–PD procedure was used to visualize and quantitate the principal CP metabolites in human urine. Fig. 4 shows a photograph of a TLC plate run with six urine samples and two authentic standard solutions. Considerable inter-patient variability was observed, particularly in the excretion of CX and PM, the probable genetic basis of which will form the subject of a separate report. The percentage dose (mean ± S.D.) eliminated in the 0–24 h urine after 0.6–1.8 g CP given intravenously to fourteen patients with various malignancies was; CP, 12.7 ± 9.3; KP, 1.0 ± 0.9; CX, 3.1 ± 3.7; NNM, 0.8 ± 0.6; and PM, 18.5 ± 16.1. Urinary excretion of total drug related material in the first day post-dose was therefore 32.6 ± 17.8% of dose. Such findings are consistent with those of investigators who have employed radioisotopically labelled CP [16–18].

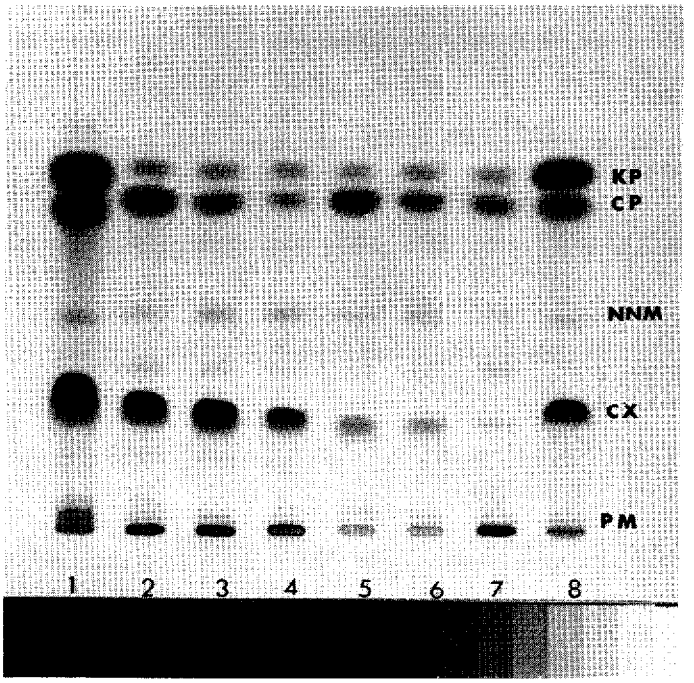


Fig. 4. Photograph of TLC plate run with six urine samples (lanes 2–7) and two authentic urinary standards (lanes 1 and 8), showing the Kodak grey scale (below). Determination of urinary excretion of each metabolite was achieved by densitometric scanning of the lanes. See text for abbreviations.

## DISCUSSION

The identity of the metabolites of CP and their interconversion is now well established [9]. One surprising weakness, however, is the paucity of data concerning the quantitative balance of the metabolites which are produced by patients given CP. Since both the therapeutic efficacy and toxicity are not attributable to the parent drug, but rather arise from metabolites, it is desirable to understand the extent to which CP is metabolized by the activation and detoxication pathways. Moreover, one of the clinical limitations of oxazaphosphorine cytotoxic drugs such as CP is the development of resistance. In certain rodent tumours, acquired resistance is the result of elevated detoxication to CX as a result of increased tumour aldehyde dehydrogenase activity [19]. There is no reason to suppose that constitutive and acquired resistance in patients does not have a similar metabolic basis. Until now such questions remain unanswered, one reason for which has been the lack of a comprehensive and simple assay for the principal metabolites of CP which did not resort to the administration of radioisotopes to patients. The TLC–PD method described in this report provides a simple and accurate means for determining CP and its four major urinary metabolites which will permit investigation into the relationship between metabolism and CP toxicity, resistance and efficacy to proceed and will allow for a better

understanding of the role of both genetic and aquired characteristics in influencing the outcome of CP therapy for the individual patient.

Unlike high-performance liquid chromatographic (HPLC) or GC assays which can have major consumable costs (columns, gases and solvents), densitometry has the virtue of being low cost with no consumables other than chart paper. The only significant costs of the TLC–PD assay arise from the purchase of preprepared HPTLC plates and from the photographic procedures. Our current estimate of the cost per sample analysed by this method is £ 0.90 in consumables and photography. Moreover, the assay will achieve what no GC, GC–mass spectrometric or HPLC assay has so far yielded, the full metabolic spectrum of cyclophosphamide without recourse to radioisotopes.

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