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Note

Gas chromatographic assay for chlorambucil and phenylacetic mustard in plasma

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Chlorambucil (CA) (I, Fig 1), a nitrogen mustard derivative of 4-phenylbutyric acid, systematic name 4-{*p*-[bis(2-chloroethyl)amino]phenyl}butyric acid, is being used for the treatment of different carcinomas [1–3] Prednimustine, a chlorambucil esterified with prednisolone, was synthesized to gain a greater antineoplastic effect with less general toxicity, using the steroid part as a carrier across the cell membrane [4]

For the determination of chlorambucil and its metabolites, several gas chromatographic–mass spectrometric (GC–MS) and high-performance liquid chromatographic (HPLC) methods have been described [1,4–12] With the method described here it is possible to measure CA and its metabolite phenylacetic mustard (PAM) in amounts down to the levels found under therapy (30–500 ng) Only a capillary gas chromatograph with a flame ionization detector was required, not a mass spectrometer

Phenylpropionic acid mustard (II), a compound very similar to CA and PAM, was synthesized as the internal standard (I S) The metabolic oxidation of CA to PAM (III) is performed by the enzymes of the β-oxidation pathway for fatty acids [13,14] If CA is also metabolized via α-oxidation to phenylpropionic acid mustard, the amounts formed are too small to be detected Therefore, the

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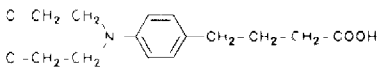
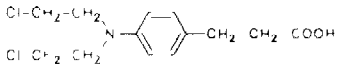


I



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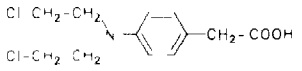
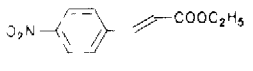


III



IV

Fig 1 Structures of (I) chlorambucil, (II) internal standard, (III) phenylacetic mustard and (IV) ethyl *p*-nitro-*trans*-cinnamate

phenylpropionic acid mustard could be used, the phenylpentanoic acid mustard did not have to be synthesized

The dihydroxy metabolites of CA and PAM could be detected after silylation with N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) with this method when added as pure compounds However, employing the extraction conditions described (essential for obtaining sufficiently clean gas chromatograms), both dihydroxy compounds remained in the acidic aqueous phase Prednimustine, a molecule too large and too labile to pass through a gas chromatographic (GC) column without decomposition, could not be detected with the described assay, an HPLC method was required for this purpose [10]

EXPERIMENTAL

Synthesis of the internal standard

The readily available ethyl *p*-nitro-*trans*-cinnamate (IV) was the starting material Catalytic hydrogenation with platinum saturated the side-chain and reduced the nitro to an amino group [15] The resulting ethyl *p*-aminophenylpropionate was allowed to react with an excess of ethylene oxide to form ethyl 4-{*p*-[bis(2-hydroxyethyl)amino]phenylpropionate [16,17] The hydroxyl groups were replaced with chlorine using thionyl chloride [18] After the ester

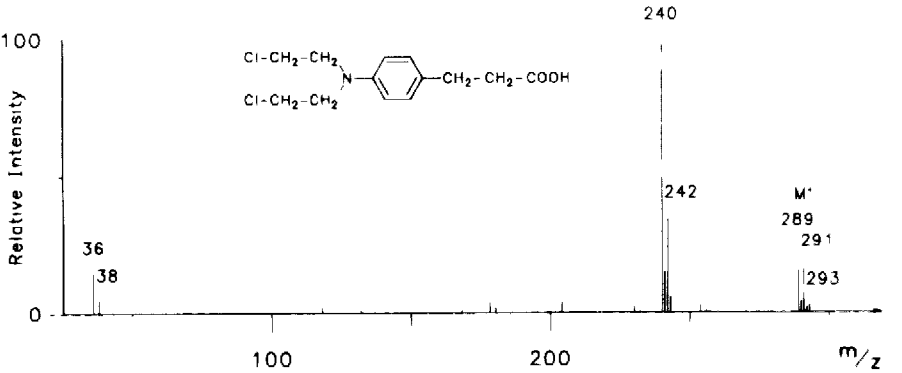


Fig 2 Electron-impact (20 eV) mass spectrum of the internal standard phenylpropionic acid mustard recorded with an LKB 2091 mass spectrometer

cleavage with concentrated hydrochloric acid, the internal standard (II) was obtained The electron-impact mass spectrum of II is shown in Fig 2

Gas chromatography

The gas chromatograph was a Carlo Erba Fractovap 4180, equipped with a cooled on-column injector and a flame ionization detector The column was a 25-m fused-silica capillary (Hewlett-Packard) with 0 17-μm 5% cross-linked phenylmethylsilicone The carrier gas was nitrogen, precolumn pressure 1 kg/cm^2, and the make-up gas was compressed air at 0 9 kg/cm^2 Signals were recorded with a Shimadzu R 112 recorder The compounds were identified by their relative retention times

Materials

Chlorambucil (Leukeran®) was generously contributed by Deutsche Wellcome (Burgwedel, F R G) Prednimustine (Sterecyt®) was provided by AB Leo (Helsingborg, Sweden) Dr D Newell donated the phenylacetic acid mustard Ethyl *trans-p*-nitrocinnamate (99%) was purchased from Aldrich Chemie (Steinheim, F R G) All other chemicals were of analytical-reagent grade

Sampling

Blood samples were obtained by venous puncture Blood samples were drawn into heparinized 10-ml tubes before and 15, 30, 60, 120, 240, 360, 480 and 720 min after drug administration The samples were cooled on ice and centrifuged Plasma was transferred into clean plastic tubes, frozen and stored at $-20\,^{\circ}$C until analysis

Work-up

Volumes of 2 ml of the plasma were spiked with 10 μl of a working solution of the internal standard (0 1 mg in 1 ml of acetonitrile) Protein was precipi-

tated with 2 ml of 1 *M* perchloric acid After being mixed for 1 min, the sample was centrifuged at 2500 *g* for 10 min The clear solution was transferred into another tube and 2 ml of dichloromethane (DCM) were added The sample was mixed and centrifuged After the centrifugation there were three layers in the tube. the top was the acidic aqueous layer, the middle layer an emulsion layer and the bottom was the organic layer The top layer was discarded A further 2 ml of DCM were added and the sample was mixed and centrifuged again This step was necessary to reduce the emulsion between the organic and aqueous layers. The organic layer was transferred into a glass tube and then evaporated to dryness under nitrogen at 45 °C

To the dry residue, 20 μl of MSTFA were added [19] The sample was redissolved by vortex-mixing for 10 s and allowed to react for 30 min at room temperature A 1-μl volume of the solution was injected into the cooled on-column injector

Chromatographic conditions

To separate the various substances, the following temperature programme was used injection temperature, 120 °C, ballistic increase to 210 °C, 5 min isothermal, increased at 5 °C/min to 260 °C, 2 min isothermal The substances were eluted at 214/215 °C (PAM), 224/225 °C (I S) and 234/235 °C (CA).

Determination of concentration

Calibration graphs for CA and PAM were established Plasma samples of 2 ml, spiked with known concentrations of CA, PAM and I S , were processed as described above For the determination of the concentrations the peak-height ratios were used With y as the peak-height ratio of substance to internal standard, the equations for the calibration graphs were $y = 0\ 0024x + 0\ 0035$ for CA (correlation coefficient $r = 0\ 998$) and $y = 0.0023x + 0.016$ for PAM ($r = 0\ 994$) The relative standard deviations were 4 9 and 5 5% for CA and PAM, respectively (measured in two samples spiked with 0 5 μg of CA and 0 5 μg of PAM each, each sample injected five times) The limit of detection in plasma was 30 ng/ml for both compounds

RESULTS AND DISCUSSION

The assay described was sensitive enough to measure the kinetics of CA and PAM in the plasma of patients treated with chlorambucil or prednimustine In Fig 3 two chromatograms are shown, obtained from the plasma of one patient before and after administration of 300 mg of prednimustine Typical concentration–time curves for CA and PAM obtained for a patient treated with prednimustine and a patient treated with chlorambucil are shown in Fig 4A and B The concentration–time curves were comparable with those obtained by an HPLC assay [10].

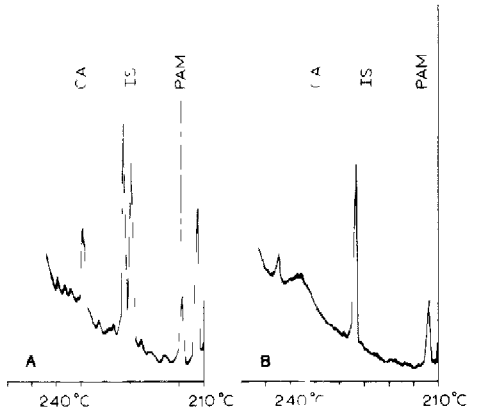


Fig 3 Chromatograms obtained from plasma of a patient treated with prednimustine (A) 4 h after and (B) before drug administration

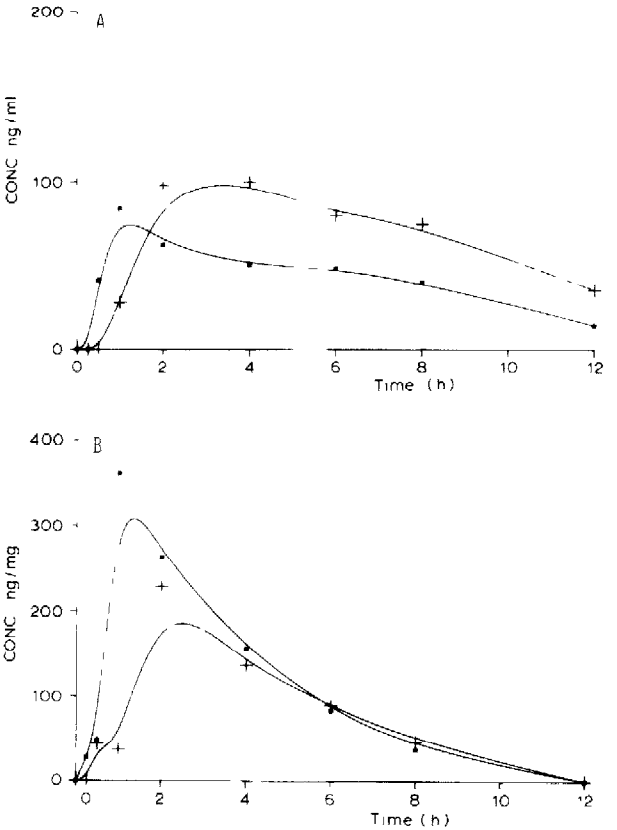


Fig 4 Concentration-time curves for (A) a patient treated with 300 mg of prednimustine (Sterecyt) and (B) a patient treated with 30 mg of chlorambucil (Leukeran) (■) CA (+) PAM

The advantages of the GC method are that the work-up for a series takes 1 5 h and is faster than that for the HPLC assay [10] A mass spectrometer as the detector could easily increase the specificity if really necessary Samples from the kinetics of one patient including the calibration graph could be worked up and measured on one day (up to eighteen samples per day) Rapid processing is desirable because of the lability of the mustard moiety

Prednimustine was too large and too labile to pass through the GC column without decomposition and could not be measured with the assay described. This is a clear disadvantage, as prednimustine is being increasingly used because of better tolerability An HPLC method will be required for the determination of prednimustine We also could not measure simultaneously the hydrolysis products of CA and PAM The hydrolysis products remained in the aqueous phase under the extraction conditions described, necessary to obtain extracts clean enough for the GC measurement This simultaneous measurement was originally intended However, we know from the results obtained by HPLC that hydrolysis products are not important if the blood samples are stored in ice immediately after sampling Plasma samples at −20 C were stable for several weeks Further, the ideal internal standard will be hydrolysed at the same rate as PAM and CA and therefore compensate for all losses

ACKNOWLEDGEMENT

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