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DETERMINATION OF CHLORTHALIDONE IN PLASMA, URINE AND RED BLOOD CELLS BY GAS CHROMATOGRAPHY WITH NITROGEN DETECTION

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SUMMARY

A sensitive and selective gas chromatographic method is described for determining the diuretic and antihypertensive drug chlorthalidone in plasma, urine and erythrocytes. Use is made of an alkali flame ionization detector (nitrogen detector), and the chlorthalidone and internal standard are chromatographed as methyl derivatives. Down to 10 ng of drug in the biological sample can be measured accurately, with a standard deviation of 5%. Because the concentration of chlorthalidone found in erythrocytes is 50–100 times higher than that in plasma, the influence of haemolysis on the plasma concentration has been investigated. In addition, a pharmacokinetic study with human volunteers revealed that the apparent concentration of the drug found in plasma can be much too low (by more than 50%), if the plasma is not separated from the erythrocytes immediately after venipuncture. Precautions to be observed to ensure correct handling of blood samples (so that results for plasma concentrations will be reliable) are stressed. The findings have application in kinetic studies on chlorthalidone.

INTRODUCTION

Chlorthalidone (Hygroton®; I in Fig. 1) is a long-acting diuretic compound, widely used in antihypertensive therapy[1]. A sensitive and accurate assay method for body fluids is necessary in order to measure the pharmacokinetic characteristics of the drug. Tweeddale and Ogilvie[2] reported a spectrophotometric method, which, however, was insufficiently sensitive for measurement of therapeutic levels of I in plasma. The use of gas chromatography (GC) in the determination of I was delayed, probably by the difficulty in forming a volatile derivative of I, which contains polar groups. Recently, extractive alkylation, with ion-pair extraction, followed by GC with electron-capture detection, led to determination of I at the nanogram level (Ervik and Gustavii[3]). However, complete analysis for a drug in body fluids must include the correct way of handling the samples, especially if variations therein affect the results. In our work, it became evident that the concentration of I found in plasma depended greatly on the method used for separating the plasma from the

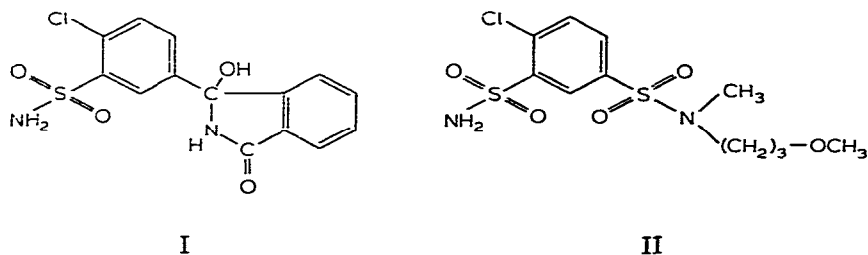I   II

Fig. 1. Structure of chlorthalidone (I) and the internal standard (II) used.

erythrocytes. Thus, we stress the proper treatment of the blood samples, as well as the GC. Use is made of the nitrogen-sensitive mode of the flame ionization detector, by means of which nanogram amounts of I (which contains two nitrogen atoms) can be measured. An internal standard (II in Fig. 1) is incorporated in the extraction procedure, and special care is taken to ensure complete recovery of the drug in the erythrocyte assay.

MATERIALS AND METHODS

Apparatus

GC was performed on a Hewlett-Packard (Avondale, Pa., U.S.A.) instrument (HP Model 5750 G), equipped with a dual nitrogen detector (alkali flame ionization detector; AFID) with a rubidium bromide crystal (HP Model 15161 A) and connected to a HP recorder (Model 7127 A).

Column. A glass column (1.8 m × 3 mm I.D.) packed with 3 % of SE-30 on Gas-Chrom Q (80–100 mesh) (Applied Science Labs., State College, Pa., U.S.A.) was used. Before being packed, the column was cleaned with chromic acid, silanized with 5 % dichlorodimethylsilane solution in toluene, rinsed with methanol and dried. The column was used for as long as peak symmetry was good (usually 3–6 months). This condition was accomplished by several pre-injections of samples containing the methyl derivatives of I and II.

Temperatures. Column, 270° (isothermal); injection port, 320°; detector, 360°.

Gas flow-rates. The carrier gas was helium (flow-rate of 60 ml/min); the flow-rates of hydrogen and air to the detector were 30 and 180 ml/min, respectively.

Operation of detector. The optimal use of an AFID necessitates exact adjustment of gas flow-rates. The distance between the collector, containing the rubidium bromide crystal, and the flame is important with respect to the sensitivity and selectivity for nitrogen-containing compounds. Also, the crystal surface must be clean and smooth. At the beginning of each day, the crystal surface was wiped with a soft brush, and the collector was lowered until maximum ionization current (maximum recorder deflection) was reached (range 10^2; attenuation 32). The detector response was monitored by an injection of 5 μl of a stock solution (500 ng of the tetramethyl derivative of I), which gave full-scale deflection at the recorder (range 10^3; attenuation 32).

Other apparatus. A shaking apparatus (Marius, Utrecht, The Netherlands) was used for extractions and where shaking is indicated. Centrifugations were carried out in a Sorvall GLC2 centrifuge. For sonication, a Bransonic 220 sonication bath (Branson, Soest, The Netherlands) was used.

Reagents

Chlorthalidone (I), 3-(4-chloro-3-sulphamoylphenyl)-3-hydroxyisoindolin-1-one, was a gift from Ciba-Geigy (Basle, Switzerland). The internal standard (II), 4-chloro-N^1-methyl-N^1-(3-methoxypropyl)-1,3-benzenedisulphonamide, was kindly supplied by Bayer (Wuppertal, G.F.R.) (courtesy of Dr. Horstmann). Stock solutions of I were prepared by dissolving 100 mg in 2000 ml of 0.067 M Sörensen phosphate buffer of pH 7.4 and diluting to the appropriate concentrations. Solutions (50 and 5 µg/ml) of II were prepared in the same buffer. Ethanol, isobutyl methyl ketone, *n*-hexane and dichloromethane (all pro analysi grade; Merck, Darmstadt, G.F.R.) were used without purification. Iodomethane (Riedel de Haen, Hannover, G.F.R.) was also used without purification. Tetrahexylammonium hydrogensulphate (Labkemi, Stockholm, Sweden) was converted into the hydroxide (0.1 M, in aqueous solution) by dissolution in ice-cold 0.2 M sodium hydroxide. All inorganic chemicals used (Merck) were of pro analysi grade. Glassware was cleaned by brushing with a commercial detergent (Dubro), followed by thorough rinsing with tap-water, distilled water and ethanol. The heparin solution (Organon, Oss, The Netherlands) contained 50 mg (5000 U) per ml.

Determination of chlorthalidone in plasma

Into a glass-stoppered conical 50-ml tube were pipetted 1 ml of plasma and 1 ml of 0.067 M Sörensen phosphate buffer of pH 7.4 containing 5 µg of II, and the mixture was extracted twice (20 min, *ca.* 250 strokes/min) with 10 ml isobutyl methyl ketone–ethanol (100:2, v/v) in the shaking apparatus. For samples expected to contain less than *ca.* 25 ng/ml, 2-ml portions were taken and 0.5 ml of 0.5 M Sörensen phosphate buffer and 0.1 ml of II solution (50 µg/ml) were used. After centrifugation for 5 min at 3000 rpm, the upper organic layers were transferred with a Pasteur pipette to another conical tube, and the drug was extracted into 2 ml of 0.1 M sodium hydroxide (15 min, *ca.* 250 strokes/min). After centrifugation, the aqueous layer was transferred to a 10-ml conical tube fitted with a screw cap having a PTFE-faced rubber liner. Next, 50 µl 0.1 M tetrahexylammonium hydroxide solution were added, and, after mixing, 5 ml of 0.75 M iodomethane in dichloromethane. The tube was shaken for 20 min at 50° (thermostatically controlled) at *ca.* 250 strokes/min. After centrifugation (5 min, 3000 rpm), the organic layer was transferred to a 10-ml tube with a finely tapered base and evaporated to dryness at 40° in a stream of purified dry air; 5 ml of *n*-hexane were added, and the tube was placed in a sonication bath for 5 min. After centrifugation (5 min, 3000 rpm), the hexane layer, containing the methyl derivatives of I and II, was transferred to another tapered 10-ml tube, and the hexane was evaporated to dryness. To the residue were added 50 µl of ethanol, and 5 µl of the solution were injected into the gas chromatograph by means of a Hamilton syringe. Each injection was made in duplicate.

Determination of chlorthalidone in erythrocytes

The erythrocytes were mixed by thorough whirling and sonicated for 15 min, then 200 µl were pipetted into a glass-stoppered 50-ml tube containing 1.8 ml of phosphate buffer of pH 7.4. Disposable Eppendorf pipettes were used, and each was washed with the buffer solution so that no red-cell material remained in it. Then II (0.1 ml, 5 µg) was added, followed by 600 mg of sodium chloride and 100 mg of

sodium fluoride, the tube contents were mixed and shaken for 15 min and the mixture was extracted and further treated exactly as described for plasma.

Determination of chlorthalidone in urine

The volume and pH of each urine fraction were measured (pH meter 22, Radiometer, Copenhagen, Denmark), and the pH was adjusted to 8.0 by adding a few drops of 5 M sodium hydroxide. To 1 ml of urine in a conical tube were added 1 ml of 0.5 M Sörensen phosphate buffer of pH 7.4 and 0.1 ml of II solution (5 μg), and the mixture was treated as described for plasma.

Preparation of calibration graphs

The concentrations of I in plasma, red cells and urine were calculated by means of calibration graphs established by adding known amounts of the drug to blank samples containing a constant amount of II. In preparing the calibration graph for the red-cell assay, intact erythrocytes (0.2 ml) were incubated for 1.5 h at 37° in 1 ml portions of the 0.067 M buffer (pH 7.4) containing known concentrations of I.

Recovery studies

The extraction yields of I from buffer and plasma into the organic layer were determined at three concentrations (25 and 250 ng/ml and 2.5 μg/ml) by adding known amounts to blank plasma and buffer of pH 7.4. After extraction, II was added, and the relative peak-area ratio after derivatization (ratio I) was compared with the value obtained after direct derivatization of I and II (ratio II). Recovery (%) was calculated as 100 times ratio I/ratio II. For II, the recovery was determined in the same way.

Taking of blood samples

Blood (sampling volume *ca.* 5 ml) was collected in heparinized glass tubes (containing 1 drop of heparin solution) and centrifuged (3000 rpm, 3 min) within 5 sec of collection. The plasma layer was then rapidly removed with a Pasteur pipette and centrifuged for 20 min (3000 rpm); the plasma was decanted into another tube. The erythrocyte layer was again centrifuged for 20 min at 3000 rpm, and the remaining supernatant liquid was discarded. The plasma and erythrocyte fractions were frozen at −20° until assayed.

In vitro incubations

In vitro incubations of I were carried out by gently agitating fresh heparinized blood (haematocrit values 0.40–0.50) from human volunteers (obtained after overnight fasting) in a water bath at 37° in air. The drug was either dissolved in a small volume of 0.1 M sodium hydroxide containing 0.3% of sodium chloride (0.1 ml for 5 ml of blood) and added directly to whole blood, or first dissolved in the plasma and subsequently added to the erythrocytes.

Influence of haemolysis on chlorthalidone plasma concentrations

Portions (100 ml) of blood were taken from three healthy subjects (age 23–26 years), after overnight fasting, under conditions preventing haemolysis, *viz.*, using a wide-bore needle and carefully heparinizing the blood[4]. The sample was freshly mixed (9:1, v/v) with I (50 μg/ml) in 0.9% sodium chloride solution and incubated for

90 min at 37°, an aliquot of the mixture being set aside before the incubation in order to obtain blank plasma. One hour after the end of the incubation, 2 ml of the blood sample was haemolysed by 3 cycles of freezing and thawing. The incubated blood was divided into portions and to each was added a small volume of haemolysate (0.025 to 0.5%, v/v). After centrifugation, the haemoglobin (Hb) concentration in each was measured relative to the blank plasma by a modified cyanomethaemoglobin method (cf. Dacie and Lewis[5]), 0.5 ml of plasma being added to 2 ml of reagent. The concentrations of I in plasma were determined in duplicate.

Identification of the methyl derivative of chlorthalidone

The methyl derivative of I was prepared in milligram amounts as follows. I (100 mg) was dissolved in 60 ml of 0.15 M sodium hydroxide in a round-bottomed flask, 25 ml of 0.5 M tetrahexylammonium hydroxide were added and, after mixing, 375 ml of 4.4 M iodomethane in dichloromethane were added, and the mixture was warmed under reflux at 40°, with vigorous stirring, for 1.5 h. The organic layer was separated and evaporated to dryness with a Rotavapor apparatus. Because of the low solubility (15 mg/dl) of the I derivative in hexane, the residue was extracted by sonication 7 times with 100-ml portions of n-hexane, after which GC showed insignificant amounts of product in the extract. After centrifugation (5 min, 3000 rpm) to precipitate the tetrahexylammonium salt, the combined hexane extracts were evaporated to dryness. A control extraction verified that the hexane layer contained only trace amounts of the insoluble tetrahexylammonium salt. The product was recrystallised from methanol and dried in vacuo over phosphorus pentoxide. This yielded white crystals (m.p. 127–128°); the reaction yield was at least 95% as judged by weighing and by comparison of the GC peak areas with those of the microgram scale reaction.

Mass spectrometry of the derivative formed was performed on a LKB 9000 mass spectrometer, with a direct inlet, and after GC on a 3% SE-30 column. High-resolution mass measurements were obtained by means of a VG Micromass 70-70F apparatus interfaced with an on-line computer system (VG Data Systems 2040; VG, Altrincham, England).

Nuclear magnetic resonance spectra of the methyl derivative dissolved in fully deuterated dimethyl sulphoxide (50 mg/ml) were obtained with a Varian 360-60-MC-NMR apparatus with tetramethylsilane as internal reference.

Infra-red spectra (in potassium bromide) were recorded with a Perkin-Elmer 257 grating spectrophotometer. The UV absorption spectrum was measured, with a Cary 118 apparatus (Varian, Palo Alto, Calif., U.S.A.), on a solution (1 mg/dl) of the compound in methanol.

RESULTS AND DISCUSSION

Gas chromatography

The AFID was used successfully in the determination of I in body fluids in the nanogram range. Recently, this type of nitrogen-sensitive detection has found increasing application in the GC of pharmaceutical compounds and has been used in determinations of low concentrations of barbiturates[6–8], phenothiazines[8,9], antiepileptic drugs[6], amphetamines and narcotic analgesics[10], diphenhydramine[11], tricyclic

antidepressants[12] and theophylline[13]; it can also be used to detect nanogram amounts of other diuretics, *e.g.*, hydrochlorothiazide and mefruside, after conversion into their methyl derivatives[14].

The tetramethyl derivative of I and the dimethyl derivative of II separated under the conditions used, the retention times being 3.2 and 2.1 min, respectively (see Fig. 2). Blank plasma, erythrocyte and urine samples from normal humans show no peaks at the retention times of I and II, or thereafter; thus, large numbers of samples can be injected sequentially. The detector response is rectilinear over a wide
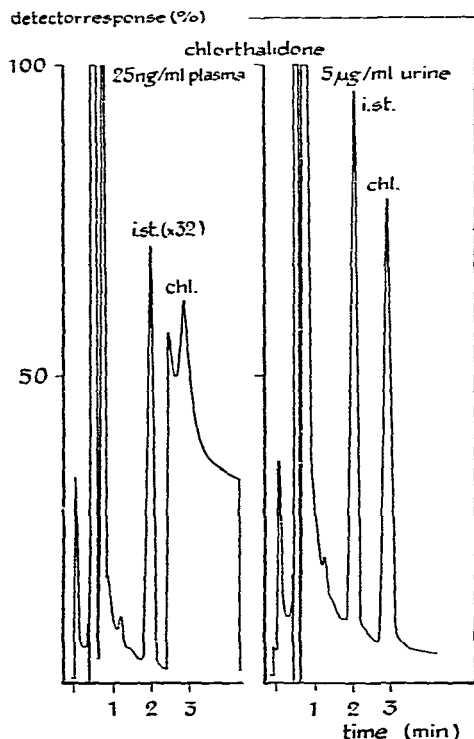

Fig. 2. Gas chromatograms showing typical concentrations of chlorthalidone in human plasma and urine. In the left-hand part chromatogram, a plasma concentration of 24.8 ng/ml was measured 100 h after an oral dose of 100 mg chlorthalidone to a healthy volunteer; in the right-hand chromatogram, a urine sample was analyzed and found to contain 5.10 μg/ml. The AFID was used in the nitrogen mode. Chl. = chlorthalidone; i. st. = internal standard (each as the methyl derivative).

concentration range, as can be seen from Fig. 3, in which a calibration graph of I added to plasma at concentrations from 20 ng/ml to 1 μg/ml is shown. The ten calibration graphs obtained were all rectilinear and passing through the origin, but varied in slope (variations about 10%) between different days. Therefore, two or three standard samples consisting of II (5 μg) and I (an amount depending on the concentration in the biological fluid, *e.g.*, 25, 100 and 500 ng for plasma) were always included in each series to check the calibration. This variation was not due to irregular extraction and/or derivatization conditions, as identical samples prepared in different runs
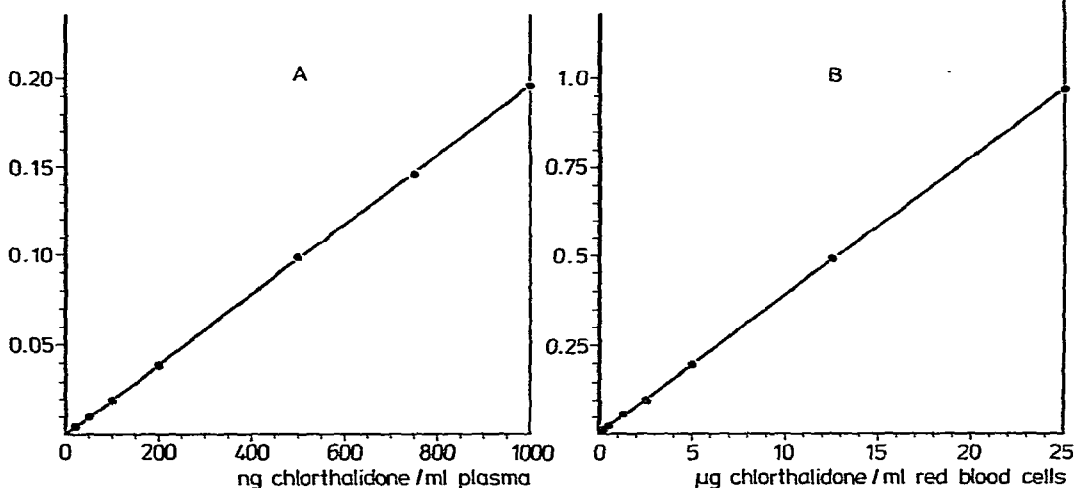
peak area chlorthalidone
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peak area internal standard

Fig. 3. Calibration graph for the determination of chlorthalidone in (A) 1 ml of plasma (concentrations ranging from 20–1000 ng/ml) and (B) in red blood cells (concentration range 0.25–25 μg/ml).

and injected into the chromatograph in the same period of time always gave identical responses, and peak-area ratios for the same sample could vary from day to day. This was attributable to differences in response of the detector to I and II because of minor changes in conditions in the detector (*e.g.*, gas flow, position of crystal) between different days.

Determination of chlorthalidone in plasma

Plasma concentrations of I down to 10 ng/ml can be accurately measured; the detection limit is smaller by a factor of 5. Standard deviations were 5% at concentrations of 1 μg/ml and 100 and 10 ng/ml (*n* = 8 at each level). The between-assay reproducibility (determinations on different days) was the same as the within-assay reproducibility. The recovery of I by extraction from plasma and phosphate buffer of pH 7.4 was 95 ± 2% (*n* = 4) at the concentrations investigated. The addition of ethanol (2%, v/v) to the isobutyl methyl ketone increased the recovery of II from 80 to 95%. The extractive-alkylation reaction necessary for the methylation of I, in which the compound is extracted as an ion-pair with the tetrahexylammonium ion and subsequently methylated with iodomethane, rapidly goes to completion under the experimental conditions, as was shown by Ervik and Gustavii[3]. By using GC–MS, we confirmed that II was completely methylated at the nitrogen atom of the free sulphamoyl group by this procedure.

Occasionally, plasma extracts were found to be contaminated by compounds of unknown origin giving rise to a "solvent peak" broader than that usually observed; excellent purification of such samples was achieved by an additional step in the isolation procedure before methylation, *viz.*, washing the 0.1 *M* sodium hydroxide layer with 10 ml of isobutyl methyl ketone-ethanol (49:1, v/v). This procedure was also advantageous in the determination of low concentrations (below 25 ng/ml) of I in

plasma by reducing the background of the gas chromatogram. (Previously, we had established that the rectilinearity of the calibration graph and the recoveries of I and II were not significantly decreased by this extra step.)

Determination of chlorthalidone in urine

Preliminary tests showed that the pH values of all urine samples to be extracted ranged from 7.40 to 7.45 if treated as described above. Calibration graphs were rectilinear, and the recoveries and standard deviations were the same as those obtained in analyses of plasma.

Determination of chlorthalidone in erythrocytes

Because of the strong binding of I to red blood cells[15–18], it was not possible to extract the drug quantitatively from this tissue with organic solvent alone. Recoveries were only 65% at a concentration of I of 10 μg/ml, and 50% at a concentration of 1 μg/ml in the red cells after two extractions with isobutyl methyl ketone-ethanol (49:1, v/v). Complete haemolysis of 0.2 ml of erythrocytes by 10 cycles of freezing and thawing in 2 ml of twice-distilled water recovered only 85% of the amount extracted from buffer solution of pH 7.4 for a red-cell concentration of 5 μg/ml. By adjusting the red cells to salt saturation by means of sodium chloride and sodium fluoride, reliable determination of I in erythrocytes was achieved, with rectilinear calibration graphs over the range 0–25 μg/ml (see, *e.g.*, Fig. 3), with standard deviations of 5% ($n = 40$). The extraction recovery in this procedure was equal to that from plasma and urine. At concentrations of I exceeding 25 μg/ml, measurements were performed by reducing the volume of erythrocytes to, *e.g.*, 0.1 ml. In calculating the concentrations of I in erythrocytes, a correction was applied for a plasma inclusion of 6% (v/v) by the packed red cells. This correction factor applies to the amount of plasma trapped in the packed cells for the relative centrifugal force and duration of centrifugation used (see, *e.g.*, Chaplin and Mollison[19]).

Stability of chlorthalidone in frozen plasma, urine and red blood cells

The concentrations of I in plasma, urine and erythrocytes from several humans remained unchanged after storage of the samples for 1 year at —20°.

Taking of blood samples and studies on the distribution of chlorthalidone between plasma and erythrocytes

During this work, it became apparent that transport of I from plasma to erythrocytes was not complete during the first few hours after ingestion of the drug, there being a great difference between the concentrations of I in plasma separated immediately after taking the blood and that in plasma from the same blood that had stood for a period. This is apparent from Fig. 4, which shows the concentrations of I in plasma from two healthy humans who had ingested an oral dose of 100 mg of Hygroton. The plasma concentrations measured after the blood had been set aside were invariably lower than those after immediate centrifugation, the greatest differences occurring in the first 10 h after administration*. This phenomenon was essentially the same with four other subjects.

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* Also, in the after-distribution period (24 h and later), there were small but distinct differences between plasma directly separated and that separated after 1 h, even if the blood was kept at 37°. This aspect may be of interest for the assay of blood that cannot be centrifuged immediately, *e.g.*, from patients outside the laboratory or clinic, and is currently being studied.
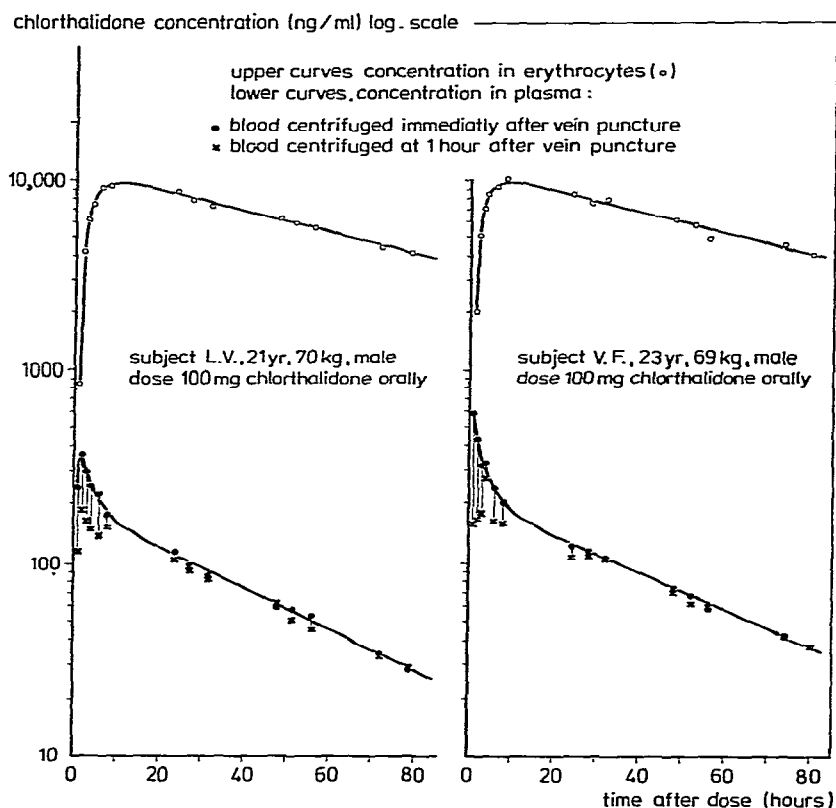
chlorthalidone concentration (ng/ml) log. scale ─────────────────────

upper curves concentration in erythrocytes (o)
lower curves. concentration in plasma :

● blood centrifuged immediatly after vein puncture
✕ blood centrifuged at 1 hour after vein puncture

subject L.V. ,21yr, 70 kg , male
dose 100 mg chlorthalidone orally

subject V. F. , 23 yr, 69 kg , male
dose 100 mg chlorthalidone orally

time after dose (hours)

Fig. 4. Lower curves: plasma concentrations of chlorthalidone *vs.* time on semi-logarithmic scale in two healthy volunteers after an oral dose of 100 mg of Hygroton. The vertical bars connect concentrations determined in plasma from the same blood samples, differing only in the period between venipuncture and centrifugation. These curves illustrate the necessity for rapidly separating the plasma from the erythrocytes in order to obtain reliable results, especially during the first few hours after absorption of the drug. Upper curves: red-cell concentrations of chlorthalidone (50–100 times greater than the corresponding plasma concentrations); the values shown are those obtained when the blood was centrifuged immediately after venipuncture.

The concentrations of I in erythrocytes are *ca.* 50–100 times higher than those in plasma (see Fig. 4). Thus, relatively large changes in plasma concentration would not be expected to affect the concentration of I in the erythrocytes significantly, except very soon after ingestion (see, *e.g.*, the low concentrations at 1 h for subjects L.V. and V.F. in Fig. 4); this was confirmed experimentally.

By *in vitro* incubations at 37°, it was shown that immediate centrifugation of the blood minimised exchange of I between plasma and erythrocytes. I was dissolved, at different concentrations, in plasma from freshly taken heparinized blood, and the plasma was again mixed with the red-cell fraction and centrifuged (3 min; 3000 rpm) within 5 sec or 5, 10, 20, 30, 60 or 150 min after mixing. From the time course of the equilibration, it was clear that distribution equilibrium was reached after 60 min (see Fig. 5). Further, in the plasma samples centrifuged at $t = 0$, the initial (added) concentrations of I were measured. This was so both when there was a high initial
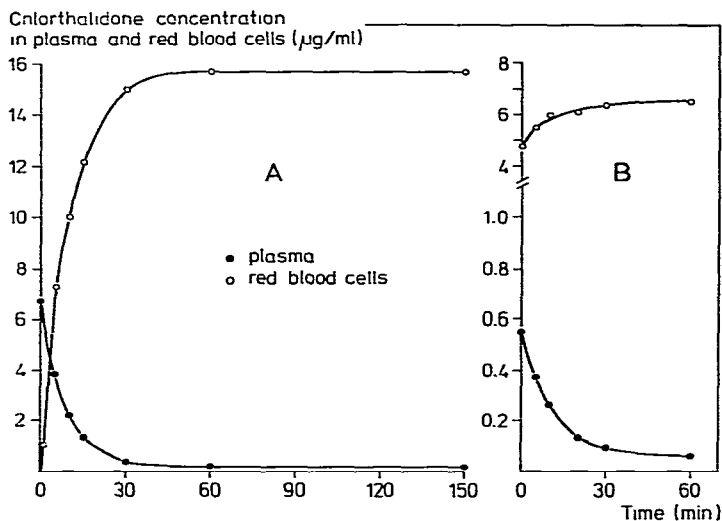
Fig. 5. Time course of *in vitro* transport of chlorthalidone from plasma to erythrocytes in two incubations of whole human blood at 37°; equilibrium was complete after 60 min. The plasma concentrations were determined after immediate separation of the plasma; thus, the measured concentrations at $t = 0$ were equal to the concentrations added at $t = 0$ (complete recovery). For further explanation see text.

gradient of plasma *vs.* red-cell concentration (Fig. 5A), and when (as *in vivo*) the erythrocytes already contained some I and more I was added to the plasma (from the gastro-intestinal tract); this *in vivo* effect was achieved by adding plasma to erythrocytes that had been pre-incubated with I (Fig. 5B). The recovery at $t = 0$ (the ratio of the concentration measured in plasma to that added to plasma) in experiments A and B was 100 and 99 %, respectively. From four of these experiments, we concluded that, in blood centrifuged immediately after venipuncture and during the shortest contact time possible in practice, the measured concentration of I in the plasma most closely resembled the *in vivo* concentration. No change in concentration was observed when plasma centrifuged for 3 min was additionally centrifuged for 20 min (after being separated from the erythrocytes). Actually, however, the plasma samples were centrifuged during this period in order to remove the buffy coat and so obtain clear samples.

To study the possible influence of heparin on the distribution of I between plasma and erythrocytes, blood samples were drawn by venipuncture from four patients receiving Hygroton, and each sample was divided into two portions, one being placed in a heparinized tube and the other in a tube containing no anticoagulant; the blood was centrifuged immediately after venipuncture. No differences were found between the concentrations of I in heparinized plasma and those in plasma without anticoagulant. Heparin also had no effect on *in vitro* incubations of I in whole blood, as was apparent from the consistency of the distribution ratio at heparin concentrations up to 10 times the normal amount (0.2 ml of heparin solution in 100 ml of whole blood).

Recently, Fleuren and van Rossum[20] investigated the equilibrium distribution of I between plasma and erythrocytes (in the *in vivo* concentration range) in a group of hypertensive patients receiving Hygroton chronically. Blood samples were drawn

by venipuncture 24 h after the last dose in order to avoid incomplete distribution, which occurs *in vivo* during the first 10–15 h (see Fig. 4) after ingestion of the drug. Had the samples been taken shortly after the dose, relatively high (non-equilibrium) plasma concentrations would have resulted (see, *e.g.*, Fig. 4).

Influence of haemolysis on chlorthalidone plasma concentrations

Because the concentration of I in erythrocytes is 50–100 times higher than that in plasma (see Fig. 4), we expected that certain degrees of haemolysis would influence the plasma concentrations. A pilot experiment showed that the graph of volume of haemolysate added to blank plasma *vs.* the spectrophotometrically determined haemoglobin (Hb) concentrations was rectilinear and reproducible when using the blank plasma as the zero point. Thus, this method is adequate for measuring different degrees of haemolysis. However, the Hb concentrations obtained are not absolute, as the assumption that the blank plasma contains no Hb was not strictly proved. Nevertheless, when blood was collected under conditions preventing haemolysis, the Hb concentration in the plasma was low [0.16–0.58 mg/100 ml (see ref. 4), equivalent to 0.099–0.36 μmol/l]. Haemolysis can be discerned with the naked eye if the plasma Hb concentration exceeds 6–12.5 μmol/l (see ref. 21). This agrees with our observations that the plasma samples with the lowest degree of haemolysis visible to the eye contained about 5 μmol/l of Hb. These considerations indicate that the Hb concentrations used (shown in Fig. 6) are not far from the absolute values (they were estimated maximally as 5 μmol/l lower than the absolute values). When the plasma concentration of I was plotted *vs.* the plasma concentration of Hb, the graph was rectilinear, with a constant slope (5.6 ng/10 μmol in Fig. 6; other experiments yielded comparable values). In Fig. 6, the concentrations of I are given as percentages of the blank plasma value (220 ng/ml for this subject). The slopes were in good agreement with the values expected theoretically on the basis of the whole-blood concentration of I, the haematocrit values and the Hb concentrations of the blood used. On the basis of three of these experiments, we decided to reject samples with a plasma concentration of Hb above 20 μmol/l, which corresponds to a degree of haemolysis of
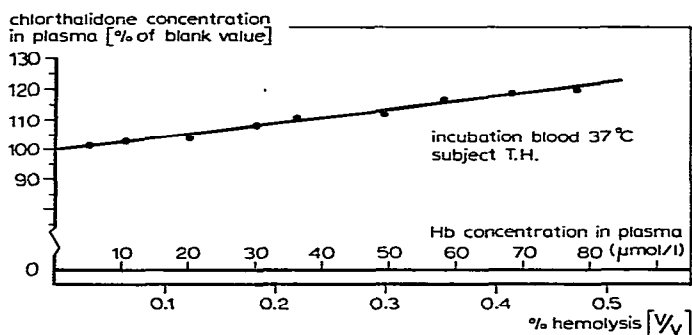

Fig. 6. Relationship between degree of haemolysis and the observed concentration of chlorthalidone in plasma after incubation of chlorthalidone (5 μg/ml) in whole human blood at 37°. The abscissa shows the degree of haemolysis as the Hb concentration in the plasma and as the percentage by volume of the blood haemolysed. The ordinate shows the plasma concentration of the drug as a percentage of the blank (unhaemolysed) plasma concentration (220 ng/ml in this experiment).

ca. 0.12 % (v/v). This degree of haemolysis could be easily discerned with the naked eye as a substantial red colour; in cases of doubt, the Hb concentration was measured. In practice, when carrying out *in vitro* incubations of I in fresh blood taken the same day, a haemolysis seldom occurred. Mostly, the plasma concentrations of Hb after the incubation were maximally 5 μmol/l higher than before. Also, in the 5-ml blood samples obtained routinely for pharmacokinetic studies, visible haemolysis almost never occurred (although, of course, this naturally depends on the skill of the person performing the venipuncture).

Identity of the chlorthalidone derivative formed in the extractive alkylation reaction

In order to confirm the identity of the methyl derivative formed, electron-impact mass spectra were recorded, and IR, UV and NMR measurements were performed. The 20-eV mass spectrum shown in Fig. 7 was obtained after direct inlet into the ion source of the LKB 9000 mass spectrometer (ion-source at 270°; trap current 60 μA), and indicates by the existence of the parent peaks at *m/e* 394 (^{35}Cl) and *m/e* 396 (^{37}Cl) that a tetramethyl derivative of I is present (the M.W. of I is 338.8). The fragmentation pattern after GC was identical. Further, high-resolution MS was performed, with the VG Micromass 70-70F mass spectrometer, of the peaks at *m/e* 363, 287 and 176. The elemental compositions corresponding with these values were: for *m/e* 363, $C_{17}H_{16}N_2O_3ClS$; for *m/e* 287, $C_{16}H_{14}NO_2Cl$; and for *m/e* 176, $C_{10}H_{10}NO_2$. The UV absorption max. was beyond 220 nm, and benzophenone absorption at and around 254 nm was absent. The IR spectrum showed one strong
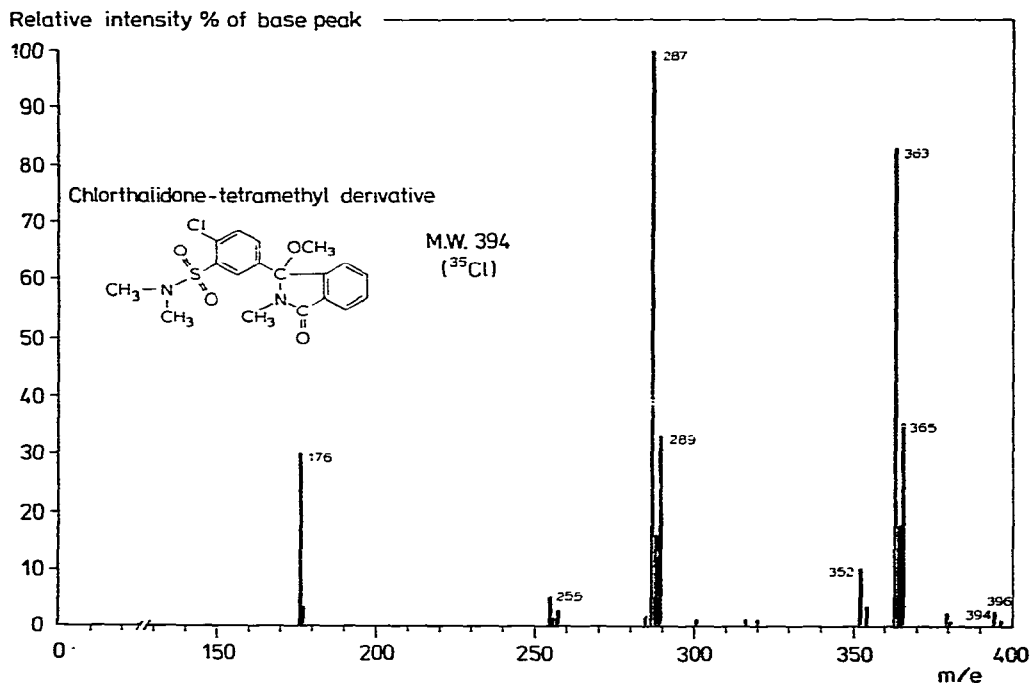

Fig. 7. Electron-impact mass spectrum (20 eV) of the tetramethyl derivative of chlorthalidone (recorded after direct inlet into the ion source of the mass spectrometer).

carbonyl absorption band at $1712\ cm^{-1}$ (5.83 μm). By comparison with reference samples of I (ref. 22), the combined UV and IR data could be attributed to the structure depicted in Fig. 7. The NMR spectra yielded values, respectively, of $\delta = 3.02$ for the six methyl-group protons bound to the sulphonamide group, $\delta = 2.92$ for the three protons bound to nitrogen in the isoindoline ring, $\delta = 3.16$ for the three protons of the methoxy-group; the seven aromatic-ring protons were between $\delta = 7.5$ and $\delta = 8.2$. The integrated values were in a ratio of 6:3:3:7. The mass spectrum shown in Fig. 7 was also given by the compound producing the peak in the gas chromatogram with the retention time of derivatized I, present in human urine after an oral dose of I. In addition, the retention time of the methylated chemical-hydrolysis product of I [G 32623:2-(4-chloro-3-sulphamoylbenzoyl)benzoic acid, kindly provided by Ciba-Geigy] differed from that of the original compound (2.8 min under the GC conditions used). This product was not formed during the extractive alkylation of I. Thus, we consider that the substance determined in biological fluids is indeed intact I and that there has been no metabolic scission of the isoindoline ring.

APPLICATIONS

The method described has been used successfully for over 2 years in pharmacokinetic studies on the fate of I in human healthy volunteers as well as in patients under antihypertensive therapy[16,20].

Because of the peculiar pharmacokinetics of I, *viz.*, different elimination half-lives of the decay curves in plasma and erythrocytes, we must emphasize again that all our results were obtained with calibration graphs for each series of determinations, with known standard solutions bracketing the concentration range of the biological samples.

The method used in the necessarily rapid separation of the plasma from the erythrocytes, in order to obtain *in vivo* plasma concentration values for I, may also be applicable to other drugs with similar characteristics of distribution between plasma and erythrocytes.

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