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QUANTITATIVE THIN-LAYER AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF THE DIURETIC AGENT 2-CHLORO-5-[4-HYDROXY-3-METHYL-2-(METHYLIMINO)-4-THIAZOLIDINYL]BENZENESULPHONAMIDE HYDROCHLORIDE IN SERUM AND URINE

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SUMMARY

Sensitive and specific thin-layer (TLC) and high-performance liquid chromatographic (HPLC) methods were developed for the determination of the diuretic agent 2-chloro-5-[4-hydroxy-3-methyl-2-(methylimino)-4-thiazolidinyl]benzenesulphonamide hydrochloride (HOE 740).

HOE 740 can be determined in serum by HPLC. The detection is performed at a very short wavelength (202 nm), resulting in a detection limit of 10 ng/ml. By TLC only urine levels that are normally high can be determined directly (the detection limit is 70 ng/ml). For the determination of the lower serum levels it is necessary to convert the drug into its dehydration product, which has a higher absorbance and gives sufficient sensitivity (the detection limit is 10 ng/ml). Serum levels determined by the two methods correlate well. Some pharmacokinetic and excretion–kinetic data were computed using two-compartment open models.

INTRODUCTION

2-Chloro-5-[4-hydroxy-3-methyl-2-(methylimino)-4-thiazolidinyl]benzenesulphonamide hydrochloride (HOE 740) is a recent diuretic agent[1], the structure of which is shown in Fig. 1.

For pharmacokinetic studies, analytical methods were required that would be suitable for quantitative and specific assays at the nanogram per millilitre level. Thin-layer (TLC) and high-performance liquid chromatography (HPLC) with UV detection were chosen for the determination of levels of the drug and the known metabolites[2] 2-chloro-5-[2,3-dihydro-3-methyl-2-(methylimino)-4-thiazolyl]benzenesulphonamide (M2) and 2-chloro-5-[2,3-dihydro-3-methyl-2-imino-4-thiazolyl]benzenesulphonamide (M3) in serum and urine.

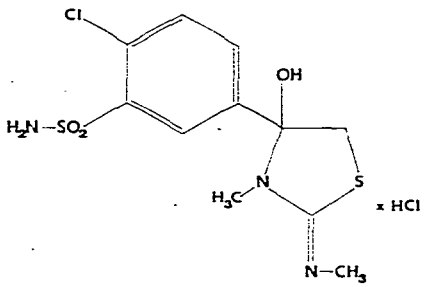

Fig. 1. Structure of HOE 740.

EXPERIMENTAL AND RESULTS

Physico-chemical properties

A knowledge of physico-chemical properties of the compound is essential for obtaining optimal conditions for its extraction from body fluids and for choosing the most suitable conditions of measurement. Therefore, some physico-chemical properties were determined.

A pK_a of 7.8, corresponding to the formation of the free base, was determined by titration. From the partition assays a second pK_a of 9.8 was calculated, relating to deprotonation of the sulphonamide.

HOE 740 is readily soluble in water below pH 6 (up to 29 g/l) and above pH 11. At pH 8.65, the minimal water solubility (0.1 g/l) corresponds to the maximal partition coefficient of 7.2 (octanol–water) (Fig. 2).

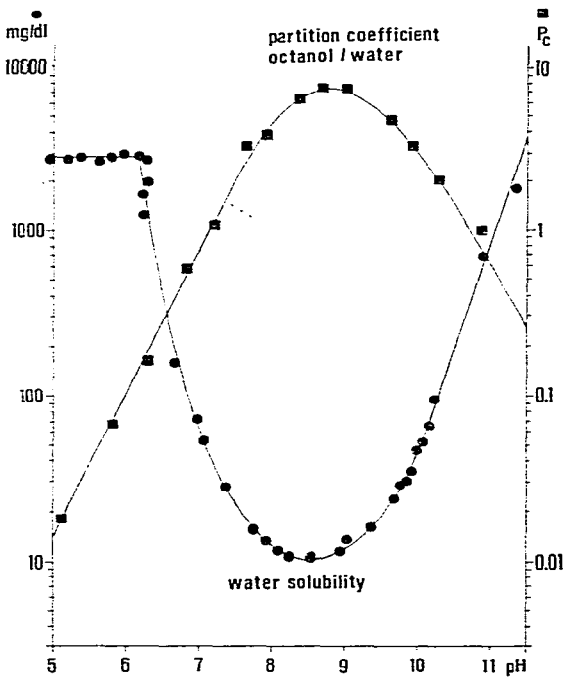

Fig. 2. pH dependence of water solubility and partition coefficient (octanol–water) of HOE 740.

In aqueous solution, UV peaks were observed at 202 nm ($E_{mol}^{1\,cm} = 45 \cdot 10^3$), 218 nm ($E_{mol}^{1\,cm} = 20 \cdot 10^3$) and 275 nm ($E_{mol}^{1\,cm} = 1.5 \cdot 10^3$), with minor peaks at 268 and 282 nm (Fig. 3). The compound shows no fluorescence.

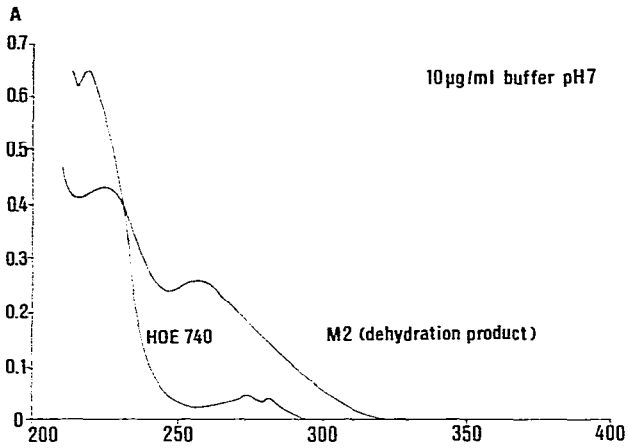

Fig. 3. UV spectra of HOE 740 and metabolite M2 (dehydration product) in buffered aqueous solutions. 10 µg/ml buffer (pH 7).

HPLC analysis in serum

By means of HPLC, the compounds can be detected at a very short wavelength (202 nm), resulting in a detection limit of *ca.* 8 ng (*ca.* 10 ng/ml).

The reagents used were ammonia solution (25%), 0.1 *N* hydrochloric acid and ethyl acetate (p.a. grade, freshly distilled), with 2-chloro-5-[4-hydroxy-2-(isopropylimino)-3-methyl-4-thiazolidinyl]benzenesulphonamide hydrobromide as the internal standard (0.1 mg/ml).

A Vortex mixer, a centrifuge, glass-stoppered tubes (*ca.* 12 ml) and conical glass-stoppered tubes (*ca.* 8 ml) were employed. The chromatograph was a Waters ALC202 with a U6K injection port and a Schoeffel SF 770 spectrophotometer, equipped with a stainless-steel column (15 cm × 4 mm I.D.). The stationary phase was Nucleosil 7-C_{18} (Macherey, Nagel & Co., Düren, G.F.R.) and the mobile phase phosphate buffer (pH 7)–methanol (1:1).

Extraction. In a glass-stoppered tube, 2 ml of serum were treated with 1 µg (10 µl) of internal standard and 100 µl of concentrated ammonia. The serum was extracted for 30 sec with 8 ml of ethyl acetate in a Vortex mixer, the phases were separated by centrifugation (5 min) and 7 ml of the organic phase were transferred into a second tube and re-extracted with 1 ml of 0.1 *N* hydrochloric acid. After addition of concentrated ammonia (0.25 ml), the mixture was re-extracted with 8 ml of ethyl acetate and 7 ml of the organic phase were transferred into the conical tubes and evaporated to dryness at 40° under a stream of nitrogen.

Chromatography. The residue was dissolved in 150 µl of the mobile phase and 100 µl were injected into the chromatograph. At a flow-rate of 1.5 ml/min the retention times (t_R) found and k' values calculated were as follows: HOE 740, t_R = 125 sec,

$k' = 1.60$; M2, $t_R = 180$ sec, $k' = 2.75$; M3, $t_R = 94$ sec, $k' = 0.96$; internal standard, $t_R = 245$ sec, $k' = 4.10$ (Fig. 4). The chromatograms were evaluated by the integrator system using an internal standard procedure.

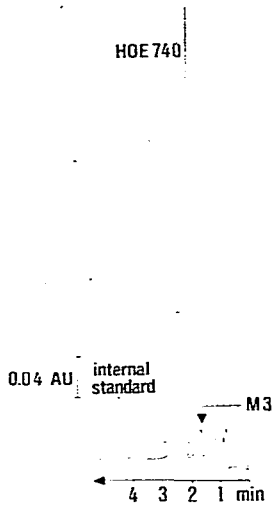

Fig. 4. Determination of HOE 740 and its metabolites in serum by HPLC. Sample taken 2.5 h after oral application of 400 mg of HOE 740. 2.35 μg/ml of HOE 740; 0.24 μg/ml of M3.

TLC analysis in urine

Densitometric *in situ* quantitation of HOE 740 at 228 nm resulted in a detection limit of *ca.* 40 ng (*ca.* 70 ng/ml), which is adequate for urine analysis (Fig. 5). Levels exceeding 10 μg/ml should be quantified at 268 nm, because calibration graphs at 228 nm become non-linear above this concentration. Metabolites were determined at 268 nm.

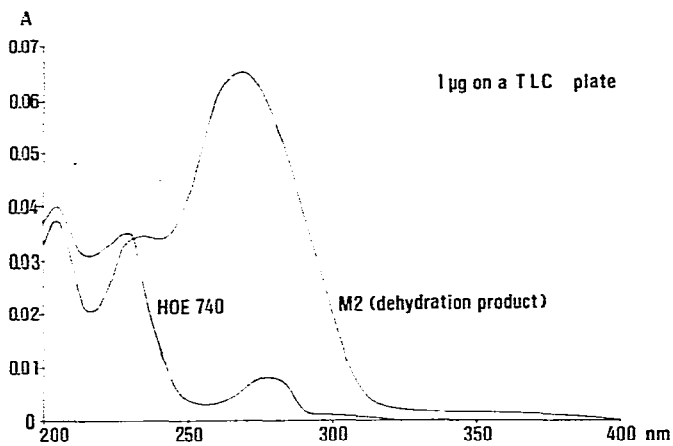

Fig. 5. *In situ* UV spectra of HOE 740 and metabolite M2 (dehydration product) on a TLC plate.

The reagents and basic equipment used were as for HPLC analysis in serum. Separation was performed on F_{254} TLC plates (Merck No. 5715) with methanol–chloroform–cyclohexane–glacial acetic acid (85:7.5:7.5:0.1) as solvent system. For quantitation a Zeiss PMQ II chromatogram spectrophotometer with a Servogor 210 (Metrawatt) recorder was used.

Extraction. In a glass-stoppered tube, 1 ml of urine was treated with 100 μl of concentrated ammonia and extracted with 5 ml of ethyl acetate. The subsequent procedure was then as described for HPLC analysis in serum.

Chromatography. The residue was dissolved in 100 μl of methanol and 50 μl were transferred on to the TLC plate using the Desaga Autospotter system. The plate was developed over a distance of 15 cm in the dark and without previous saturation. The R_F values were as follows: HOE 740, 0.6; M2, 0.4; M3, 0.3 (Fig. 6).

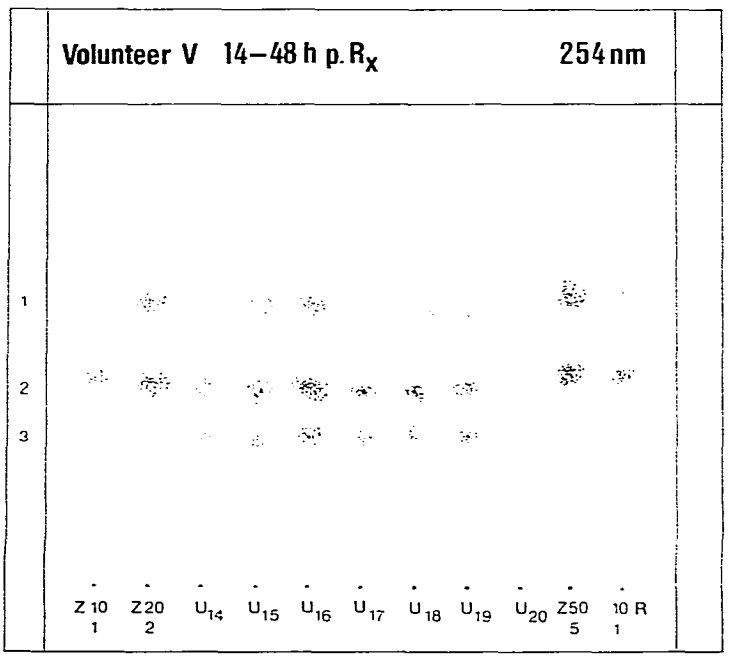

Fig. 6. Determination of HOE 740 and its metabolites in urine by TLC. Samples taken 14–48 h after oral application of 400 mg of HOE 740. 1, HOE 740; 2, M2; 3, M3. Z, Known admixtures; U, samples; R, pure substances.

Measurements on the plates were carried out in the reflectance mode in the direction of the solvent flow with a 3.5 × 2 mm slit, scanning speed 30 mm/min and paper speed 120 ml/min. Peak areas of the compounds of interest were evaluated and the compounds were quantified by means of a calibration graph based on parallel analysis on the same plate of known amounts admixed with human urine. During processing and also on the plate, *ca.* 2% of HOE 740 was converted into the dehydration product, and appropriate corrections were therefore applied.

TLC analysis in serum

The low levels of the compound that occur in serum can be quantified after forming the dehydration product and measuring at 268 nm (detection limit 10 ng/ml) (*cf.*, Fig. 5).

The reagents were as for HPLC analysis in serum, with 2-chloro-5-[3-ethyl-2-(ethylimino)-4-hydroxy-4-thiazolidinyl]benzenesulphonamide hydrobromide as the internal standard (0.1 mg/ml). The equipment was as for TLC analysis in urine.

Extraction. The extraction procedure was the same as for HPLC in serum. In the hydrochloric acid phase the drug and the internal standard were converted into the dehydration products by heating for 2 h at 95°.

Chromatography. TLC was carried out as for urine, and the R_F values were as follows: dehydration product of HOE 740, 0.4; dehydration product of the internal standard, *i.e.*, 2-chloro-5-[2,3-dihydro-3-ethyl-2-(ethylimino)-4-thiazolyl]benzenesulphonamide, 0.6.

Evaluation of the chromatograms was carried out as for TLC analysis in urine.

Statistical analysis

The reliability of the methods was tested according to the recommendations on quality control in clinical chemistry[3,4], which suggest the quality criteria listed below.

Specificity. In each instance, interferences from serum or urine constituents were absent. HPLC analyses indicated that the dehydration product was not found in serum. Therefore, no loss of specificity occurred in the TLC determination in which the compound was converted into its dehydration product before analysis.

Precision. Spiked samples were divided up and determined six times on different days. By correlating the standard deviations (S.D.) with the amounts found, the precision could be expressed as follows: HOE 740 in serum (10–1500 ng/ml) (TLC or HPLC), S.D. = 4 ng/ml + 5% of amount; HOE 740 in urine (100–10 000 ng/ml) (TLC), S.D. = 28 ng/ml + 3.5% of amount; M2 in urine (100–2000 ng/ml) (TLC), S.D. = 12 ng/ml + 12% of amount.

Accuracy. The accuracy was evaluated from the determinations on spiked samples used for the calculation of precision. In each instance, the amounts found were correlated with the amounts added. Between 97 and 101% of the amounts added were found, with regression coefficients between 0.996 and 0.999.

Sensitivity. Sensitivity, expressed as approximately 2 × S.D., was as follows: HOE 740 in serum, 10 ng/ml; HOE 740 in urine, 70 ng/ml; M2 in urine, 35 ng/ml.

Comparison of the methods

Parallel analyses of serum samples from human trials were performed by TLC and HPLC. The results of both methods correlated well (Fig. 7).

Pharmacokinetics

HOE 740 was administered orally in doses of 12.5 mg to three healthy male volunteers (volunteer I, age 42, height 1.73 m, weight 72.0 kg; II, age 38, height

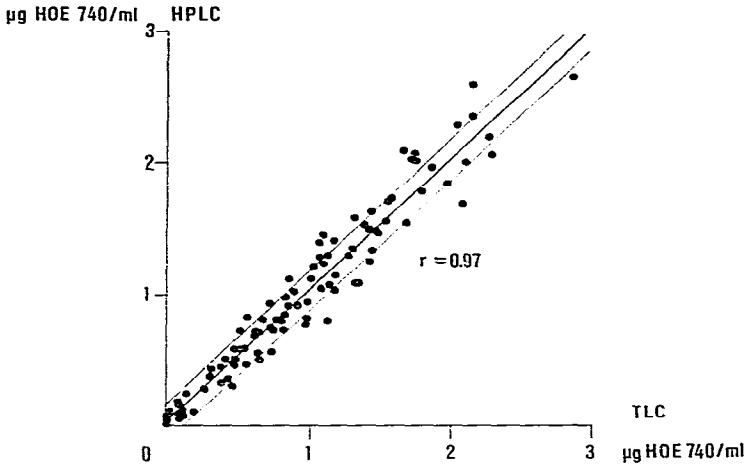

Fig. 7. Parallel analyses of human serum samples by TLC and HPLC. HPLC: $\bar{c} = 0.96\,\mu g/ml$; S.D. $= 0.154\,\mu g/ml \approx 16\%$ of $\bar{c}$. TLC: $\bar{c} = 0.92\,\mu g/ml$; S.D. $= 0.158\,\mu g/ml \approx 17\%$ of $\bar{c}$. $C_{HPLC} = (1.00 \pm 0.02) \cdot C_{TLC} + (0.04 \pm 0.03)\,\mu g/ml$ of HOE 740. $C_{TLC} = (0.95 \pm 0.02) \cdot C_{HPLC} + (0.01 \pm 0.03)\,\mu g/ml$ of HOE 740.

1.72 m, weight 69.4 kg; III, age 34, height 1.75 m, weight 85.9 kg)*. Serum and urine levels were determined and the pharmacokinetic profiles were calculated by means of computer programs (two-compartment open models)[5,6]. This is demonstrated by the serum and excretion kinetics obtained from the three volunteers in Figs. 8 and 9.

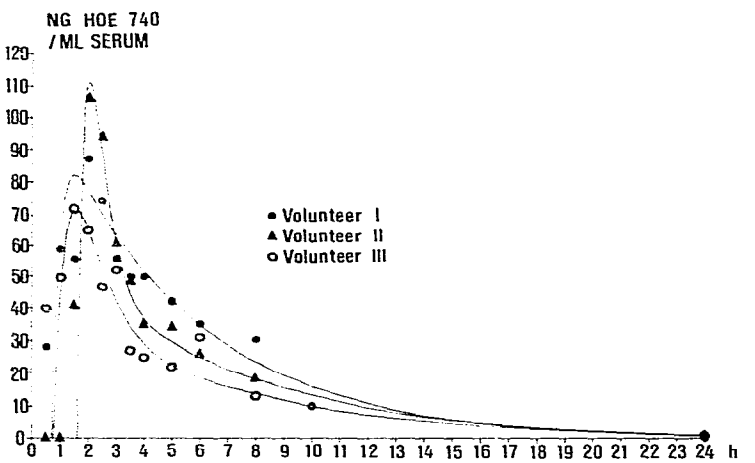

Fig. 8. Serum pharmacokinetics after oral application of 12.5 mg of HOE 740 to volunteers I (●), II (▲) and III (○).

* These studies were performed by Drs. W. Rupp and E. E. Dagrosa, Hoechst AG, Medica Department.

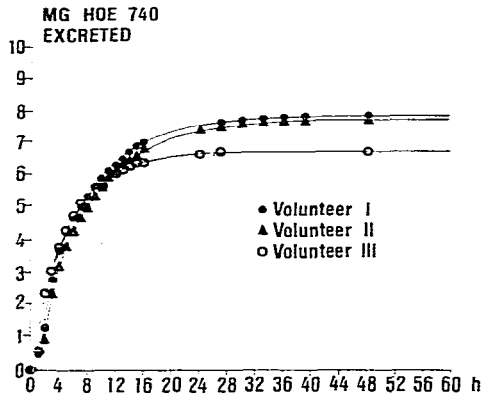

Fig. 9. Cumulative renal excretion after oral application of 12.5 mg HOE 740 to volunteers I (●), II (▲) and III (○).

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