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QUANTITATIVE DETERMINATION OF ACEMETACIN AND ITS METABOLITE INDOMETACIN IN BLOOD AND PLASMA BY COLUMN LIQUID CHROMATOGRAPHY

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SUMMARY

A column liquid chromatographic (LC) method using UV detection for the determination of acemetacin and its metabolite indometacin in blood is described The lower detection limit for both compounds is ca 25 μg/l, the precision (coefficient of variation) is 6% for acemetacin and 10% for indometacin The method is also suited for determination of both compounds in plasma, precisions in this case are even better than for blood, i e around 3% for both acemetacin and indometacin Blood samples of three volunteers who had received 90 mg of acemetacin orally were analysed using the new method and very good agreement with results from a thin-layer chromatographic/fluorescence method was found

INTRODUCTION

Acemetacin is a new potent non-steroidal anti-inflammatory compound which is used for the treatment of arthritis and rheumatic diseases. Measurements of levels of both acemetacin and its active metabolite indometacin in blood or plasma are required for detailed studies on the pharmacokinetics of acemetacin in man.

Analytical methods for the determination of acemetacin and indometacin have already been developed, e g. measurement after thin-layer chromatographic (TLC) separation and detection [1] with a typical lower detection limit of ca. 50 μg/l. After clinically effective doses of acemetacin, concentrations of acemetacin at some time points are so low that detection and quantification is difficult by the TLC method This need for lowering detection limits and the possibility of automation was the reason for the attempt to develop an LC method for determination of both compounds in blood and plasma, respective-

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ly. In the case of compounds with high extinction coefficients, such as indoles, the combination of LC and a sensitive UV detector should make it possible to reach a lower detection limit of ca. 10 μg/l.

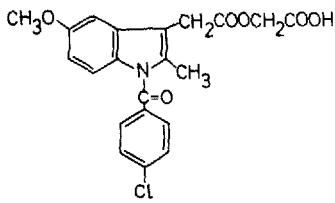
The present paper describes a column LC method that has high sensitivity, selectivity and precision for the determination of acemetacin and indometacin in human blood and plasma. It is shown how the method is used for measuring the distribution of both compounds between plasma and blood cells, it is further shown how acemetacin and indometacin can be determined in blood of volunteers given 90 mg of acemetacin and how the results are checked for their reliability by comparison with results from the above-mentioned TLC method with fluorescence detection

EXPERIMENTAL

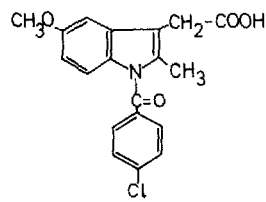
Drugs and reagents

For use as standards, acemetacin, [1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetoxy]acetic acid, and indometacin, 1(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid, were taken from production batches at Troponwerke and checked for identity and purity by the Quality-Control Department. The chemical structures of the drugs are shown in Fig 1.

Retard capsules of acemetacin (Rantudil Retard) were obtained from the Galenic Department (Troponwerke) and contained 90 mg of acemetacin. Solvents and reagents used were of reagent grade and were obtained either from Merck (Darmstadt, F R.G) or Baker Chemikalien (Gross-Gerau, F R.G).



Acemetacin



Indometacin

Fig 1 Chemical structures of acemetacin and indometacin

Chromatography of blood and plasma samples

The HPLC apparatus used in this study was a Hewlett-Packard 1090 with an HP 79846 A injector and an HP 79847 A automatic sample changer. An HP FPD 79881 A UV filter detector or HP DAD 79880 A diode array detector, both at a wavelength of 254 nm, were used for detection.

Samples from volunteers, and reference samples which had been stored at −20°C were heated to 37°C by 10 min of incubation The whole analysis was done in double determinations A 1-ml sample in a 10-ml glass-stoppered centrifuge tube was mixed with 1 ml of KH_2PO_4–H_3PO_4 (0 15 mol/l, pH 3) and 5 ml of diethyl ether, shaken for 15 min and centrifuged for 5 min at 4°C and 2000 *g* (3000 r p m). A 3-ml aliquot of the supernatant diethyl ether phase was placed in a 10-ml centrifuge glass. Another portion (4 ml) of diethyl ether was added to the sample, extracted as above, and 4 ml of the diethyl ether

phase was taken off and added to the first fraction The diethyl ether was evaporated at 37°C by a stream of nitrogen, the walls of the centrifuge glass were washed with 1 ml of methanol and the solvent was again evaporated using a stream of nitrogen

The dry samples were dissolved in 150 µl of a mixture of KH_2PO_4—H_3PO_4 buffer (0.02 mol/l, pH 4 5)—methanol (1·1), centrifuged and transferred into conical crimp-top vials (Chromacol 2CV, WGA, Pfungstadt, F R G) with removable conical inserts (No. 10381, Chrompack, Müllheim, F R G) and crimp caps (No. 5080-8766, Hewlett-Packard, Böblingen, F R G.). For analysis, 25 µl were injected by the automatic injection system

The mobile phase consisted of KH_2PO_4—H_3PO_4 buffer (0 02 mol/l, pH 4 5)—methanol (45:55), both degassed with helium. The flow-rate was 1 4 ml/min

The column (Bischoff Hyperchrom SC, 125 × 4.6 mm I.D.) was filled with a RP-18 phase Nucleosil 5 C_{18} (5 µm) and maintained at a temperature of 40°C Typical retention times for this set-up were 6 9—7 0 min for acemetacin and 8.7—8 9 min for indometacin. Both compounds were detected at 254 nm The above-described final procedure was used for all the following experiments

Calibration curves for the determination of acemetacin and indometacin in plasma

Plasma was spiked with both acemetacin and indometacin in concentrations from 25 to 1000 µg/l and extracted either twice with diethyl ether according to the above scheme, or once by adding 10 ml of diethyl ether to the buffered plasma sample, taking off 8 ml and processing as above

Measurements were done in double determinations and mean relative ranges of these double determinations are given

The relationship between detector signal and concentration was investigated to find out if it could be described satisfactorily by a linear regression line

Distribution of acemetacin and indometacin in plasma and blood cells

Fresh blood from volunteers (Institut für Transfusionsmedizin, Köln-Merheim, F R G) was put into 10-ml plastic tubes (Sarstedt, Nümbrecht, F.R.G.) to mimic actual sample treatment and each sample was spiked with acemetacin and indometacin at concentrations of 200 µg/l From half of the samples, plasma was prepared by centrifugation and both blood and plasma were stored at −20°C. After a few days, the samples were analysed according to the above-described method.

From the results, it can be judged whether determination from blood and from plasma was likewise applicable, precisions for both methods can be determined

Sample collection from volunteers

From a study on bioavailability of a Rantudil retard formulation (clinical study No 495, Troponwerke), where blood levels of acemetacin and indometacin had been determined by the above-mentioned TLC method, blood samples of three volunteers were taken and analysed in double determinations by the LC method Thus, it was tested whether metabolites formed from acemetacin or indometacin during body passage in man interfered with the LC

determination and how results from both methods correlated with each other

TLC determination of acemetacin and indometacin in blood of volunteers

Determination was done according to Dell et al. [1], with the exception that system a was abandoned and TLC plates were developed twice with the system chloroform—ethyl acetate (75 25) (system d) and once with the system ethyl acetate—isopropanol– water (65 24 11) (system b).

RESULTS AND DISCUSSION

Different plasma pH values and the solvents toluene, dichloromethane and diethyl ether were investigated for their influence on extraction recoveries; single and repeated extractions were tested

For a general overview of separation conditions and peak forms, several parameters were varied stationary phase (column material) RP-2 (LiChrosorb, Merck), RP-8 and RP-18 (both Nucleosil, Macherey-Nagel, Düren, F R G), mobile phase buffers, pH 3—9 with 40—60% methanol as organic modifier, wavelength for UV detection at 340—210 nm.

It was found that the RP-18 phase had the right polarity for separation; RP-2 and RP-8 were too polar, acemetacin and indometacin therefore giving broad peaks and long retention times Increasing the content of methanol as organic modifier did not improve column performance.

The fine adjustment of peak form and separation conditions was then done with diethyl ether-extracted samples by variation of the ionic strength of the phosphate buffer (KH_2PO_4—H_3PO_4) from 0.01 to 0 05 mol/l; the pH value of the phosphate buffer from 4 to 6, the content of methanol from 40 to 60%

Only a small region of pH (4—6) was useful to achieve good separation of acemetacin and indometacin from each other and from plasma constituents More acidic mobile phases gave good separations but very long retention times, whereas the reverse was the case with more basic systems

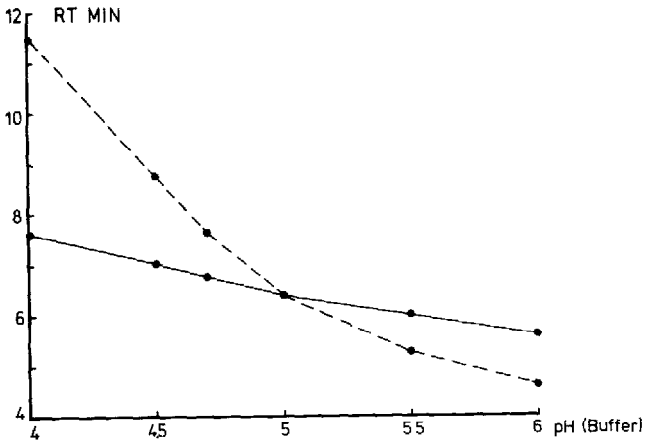


Fig 2 LC separation of acemetacin (solid line) and indometacin (dashed line) on a RP-18 column, example of crossover of retention times (RT) as a function of pH of the mobile phase Mobile phase 55% methanol, 0 02 mol/l KH_2PO_4 adjusted to pH 4—4 65 by phosphoric acid and to pH 5—6 by sodium hydroxide

Fig. 2 shows the dependence of retention times of acemetacin and indometacin on the pH value of the mobile phase in the narrow pH range 4—6. At pH 4 the first component eluted is acemetacin, at pH 5 both compounds elute together. On increasing the pH still further, indometacin is now eluted faster than acemetacin. Retention times of both compounds decrease as the pH is increased, but this decrease is much faster for indometacin, so that crossover of retention times occurs The reason for this strong change in polarity for indometacin may lie in the dissociation of the carboxyl group, the pK for indometacin (4.23) being in this range, whereas acemetacin has a pK of 2.86 [2] This strong dependence of retention time on pH value has an advantage for the chromatography of plasma and blood extracts By small changes in the pH of the mobile phase, the retention times of acemetacin and indometacin can be fitted to the "background", which is coextracted from the biological matrix

Fig 3 shows how in an extract of blood made up from reference and

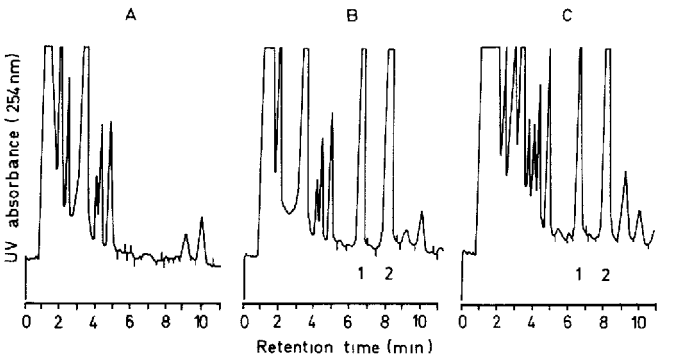


Fig 3 Quantitative determination of acemetacin and indometacin in blood by LC separation on a RP-18 column with methanol—0 02 mol/l KH_2PO_4 pH 4 5 (55 45) as mobile phase Comparison of three samples with identical attenuation (A) Blood blank, (B) blood spiked with 400 μg/l acemetacin and 600 μg/l indometacin, (C) blood of a volunteer 2 h after dosage containing 160 μg/l acemetacin and 205 μg/l indometacin Peaks 1 = acemetacin, retention time 6 90–6 93 min, 2 = indometacin, retention time 8 56—8 61 min

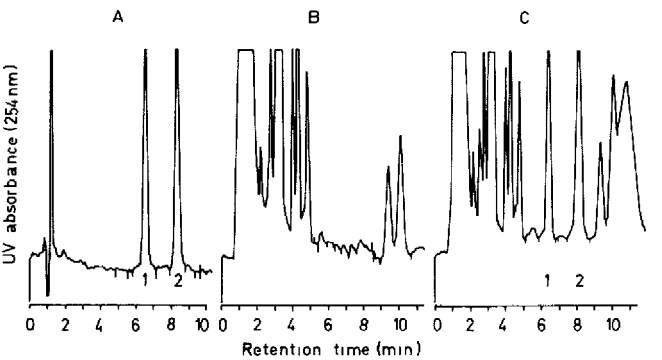


Fig 4 Quantitative determination of acemetacin and indometacin in plasma by LC separation on a RP-18 column with methanol—0 02 mol/l KH_2PO_4 pH 4 5 (55 45) as mobile phase Comparison of three samples with identical attenuation (A) Standard, 25 ng of acemetacin and 25 ng of indometacin, (B) plasma blank, (C) plasma spiked with 200 μg/l acemetacin and 200 μg/l indometacin Peaks 1 = acemetacin, retention time 6 91—6 95 min, 2 = indometacin, retention time 8 75—8 85 min

volunteer samples by choice of proper separation conditions, acemetacin and indometacin were nicely separated from the background. This is shown in Fig 4 for plasma references

Calibration of the method can be achieved by use of references made from plasma or blood spiked with only one concentration The chromatographic peaks after UV detection are expressed in signal-height counts by using the corresponding option of the HP integrator The dependence of this signal height on the concentration of acemetacin and indometacin in plasma or blood references can be described by a linear relationship. As an example, calibration lines using spiked plasma samples (25—1000 μg/l) followed the equations $y = 41\ 16x + 173.69$ with $r = 0\ 998$ for indometacin, and $y = 48\ 25x - 67\ 50$ with $r = 1\ 000$ for acemetacin, where y was the height count of the integrator and x the concentration in plasma references

Double determinations gave narrow mean ranges of 7% for acemetacin and 8% for indometacin Recoveries were high, being 89% for acemetacin and 96% for indometacin If diethyl ether extraction was done only once, slightly lower recoveries of 88 and 91% were achieved We sticked, however, to the two-fold extractions to yield a more reliable coefficient of variation (C.V).

The lower detection limit for both substances is certainly below 25 μg/l of blood or plasma, which was the lowest concentration tested In both cases, a clearly visible and measurable peak resulted with a signal-to-noise ratio of 3 1.

The distribution of acemetacin and indometacin between plasma and blood cells is in favour of the plasma Of 200 μg/l added to blood, 175.4 ± 10 3 μg/l (88% recovery, C.V = 5 9%) acemetacin and 202 8 ± 20.4 μg/l (101% recovery, C.V = 10 1%) indometacin were found, which corresponds to the above-described recovery from plasma In plasma made up from these blood samples 268.8 ± 9.9 μg/l (C.V. = 3%) acemetacin and 300 3 ± 5 2 μg/l (C.V = 1 7%) indometacin were recovered. On the assumption of an average haematocrit of 0.5, the greater part, i.e. 67% acemetacin and 75% indometacin, is dissolved in

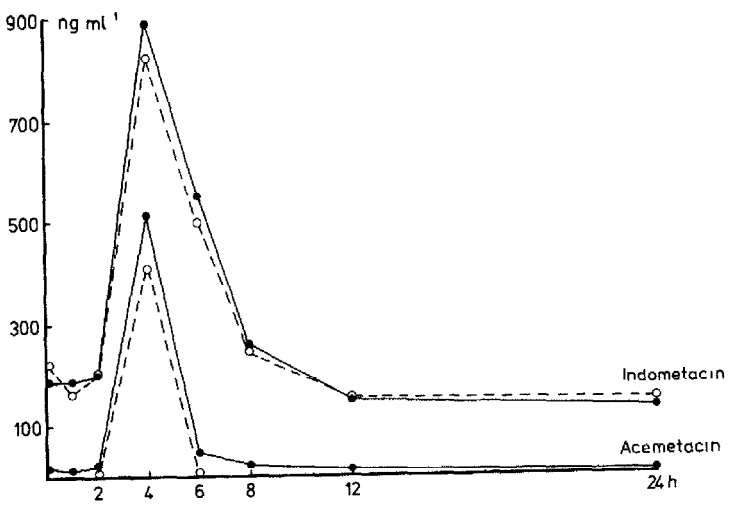


Fig 5 Quantitative determination of acemetacin and indometacin in blood by LC (closed symbols) Comparison with results of a TLC method (open symbols) Blood samples from Tropon study KF-495 (volunteer, G E)

plasma in free and bound form whereas the rest is contained in the blood cells For this reason and because of the lower C V , plasma analysis should be preferred.

Analytical determinations used for acemetacin and indometacin in previous studies [1] were by TLC and fluorescence, whereby interfering substances (e g metabolites) were separated by TLC, thus making the determination specific for acemetacin Indometacin was investigated by many groups by the same or a modified method [3—6]. Its determination by LC [7—9] or GC [10] did not give divergent data. Pharmacokinetic data of indometacin, e g. half-life, renal clearance, bioavailability, which were found by a TLC—fluorescence method, did not differ from data in the literature from other analytical procedures Blood samples of volunteers could be analysed with double determinations, being in the mean range of 12% for acemetacin and 9% for indometacin A typical example is shown in Fig. 5 As can be seen from this figure, the course of the blood level can equally well be described by results from both methods. The absolute contents by TLC, however, are always 10– 20% lower than by LC. This was confirmed by data from two more volunteers (not shown) It can also be seen that concentrations below 50 μg/l can still be measured reliably by LC, whereas TLC already has its lower detection limit in this range.

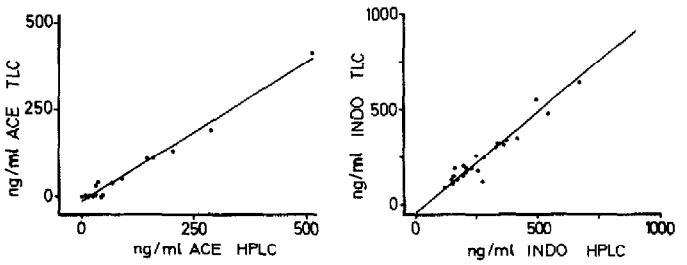


Fig 6 Quantitative determination of acemetacin (ACE) and indometacin (INDO) in blood Correlation of results from a LC and a TLC method 24 blood samples from three volunteers (G A , H K , G E) in Tropon study KF-495 Correlation coefficients for acemetacin, 0 988, for indometacin, 0 976

The correlation between LC and TLC from all 24 blood samples from the three volunteers is given in Fig. 6. The linear correlation holds in the range 0—500 μg/l for acemetacin and 0—1000 μg/l for indometacin; higher concentrations were not tested This good correlation means that conclusions drawn from blood-level studies in earlier investigations, where analyses were done by TLC, can be compared to conclusions from data by the new HPLC method. Since both methods give virtually identical results in an analytical sense, it is very likely that they both describe true blood levels.

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