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SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF INDOMETHACIN IN HUMAN PLASMA

PHARMACOKINETIC STUDIES AFTER SINGLE ORAL DOSE

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SUMMARY

A sensitive high-performance liquid chromatographic assay for the specific determination of indomethacin at concentrations down to 20 ng/ml in human plasma is described.

This method has been applied to investigate the disappearance of indomethacin from plasma of ten subjects following the intake of two formulations (Indocid® and generic form). An initial half-life of $1.32 \pm 0.44\ h^{-1}$ was found which is in good agreement with other findings, but the terminal phase was much longer ($13.6 \pm 6.9\ h^{-1}$) than previously reported. There is no difference between the two galenic forms ($p < 0.001$).

INTRODUCTION

Indomethacin [1-(*p*-chlorobenzoyl)-5-methoxy-2-methyl-indol-3-acetic acid] has analgesic, anti-inflammatory and antipyretic actions. It is used to relieve the painful symptoms of ankylosing spondylitis and osteoarthritis and to relieve the pain and swelling in gout and rheumatoid arthritis.

Plasma concentration curves of indomethacin with time vary considerably after the same oral dose in different patients [1]. There is thus a definite need for pharmacokinetic investigations with indomethacin in humans as a function of dose and galenic forms, using specific and sensitive methods of analysis. Various methods have been used for its determination based on thin-layer chromatography [2], gas chromatography (GC) with electron-capture detection after derivatization [3—5], radioimmunoassay [6] and fluorimetry [7, 8]. Most of these methods are rather unspecific (fluorimetry), time-consuming or not suitable for routine analysis.

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Recently, several methods for the determination of indomethacin by high-performance liquid chromatography (HPLC) have been reported [9—13] but they are not sufficiently sensitive (detection limit 0.1 μg/ml [9] for determining plasma concentrations of the drug, after the oral administration of therapeutic doses to humans, in the slow terminal elimination phase (plasma concentration about 20—50 ng/ml at 24 h after dosing).

This paper describes an HPLC procedure for the determination of very low concentrations of indomethacin in plasma, involving deproteinization and one-step extraction with ethyl acetate at pH 3.

EXPERIMENTAL

Chemicals

Indomethacin (lot KB 104) was generously supplied by LFPG (Marly, France). Phenylbutazone, used as internal standard, was kindly furnished by Ciba-Geigy (Basel, Switzerland) (lot AN 7311/5). Acetonitrile for UV was obtained from Fisons (Loughborough, Great Britain). Ethyl acetate was obtained from Carlo-Erba (Milan, Italy). Citrate buffer (pH 3), anhydrous sodium sulfate and acetic acid were obtained from Merck (Darmstadt, G.F.R.). All other chemicals used were analytical or LC grade.

Vessels

All glassware was washed twice with re-distilled water and methanol and dried overnight at 100°C before use.

Chromatography

The HPLC system consisted of a solvent delivery system (Altex-Chromatem 380; Touzart et Matignon, Paris, France) and a 50-μl fixed-volume loop injector (Rheodyne 7010, Berkeley, CA, U.S.A.). A 25 × 0.46 cm I.D. reversed-phase column was packed with 10-μm Partisil ODS-2 (Whatman, Clifton, NJ, U.S.A.), and was fitted with a 6 × 0.46 cm I.D. precolumn packed with Co-Pell ODS (C_{18} pellicular 37—50 μm, Whatman). The downflow slurry packing technique with slamming process [14] using a constant-pressure pneumatic amplifier pump (Haskel, Burbank, CA, U.S.A.) was used to prepare the column. Slurry solvent was *n*-butanol and isooctane was used as pumping fluid (pressure 350 bars). A variable-wavelength UV detector (Pye-Unicam, Cambridge, Great Britain) was used at 250 nm. A recorder (Linear 1201, Linear Inc., Irvine, CA, U.S.A.) was linked to the detector and a chart speed of 20 cm/h was used.

The mobile phase for isocratic chromatography was a mixture of acetonitrile and 0.1 *M* acetic acid (60:40, v/v). The chromatographic system was operated at ambient temperature at a flow-rate of 1.8 ml/min (linear velocity = 0.5 cm/sec) and a pressure of 70 bars. The mobile phase was degassed by ultrasonic treatment and by a helium stream during the determination. After use, the column was washed for 10 min with water and 30 min with methanol (2 ml/min) to prolong its life.

In vivo study

Plasma was collected by venous puncture in heparinized vials from ten

healthy volunteers (six males and four females, 25.7 ± 2.3 years) who had first been randomized and then received a single oral dose of indomethacin (75 mg, about 1 mg/kg) in two formulations (Indocid® or generic indomethacin, LFPG). At least one week intervened between the administration of any two formulations to any subject. Subjects were fasted for 12 h before administration. Repeated blood samples were obtained during the following 34 h (0, 0.5, 1, 2, 3, 4, 6, 8, 10, 24, 34 h) and were centrifuged within 10 min at 1200 *g* (4°C) to obtain plasma (stored at −30°C until analysis).

Assay in plasma

One milliliter of patient plasma and 100 μl of a phenylbutazone solution (10 μg/ml, prepared daily from a stock solution of 1 mg/ml in methanol) were mixed with 1 ml of acetonitrile (Vortex). After 10 min, precipitated protein was removed by centrifugation (10 min, 1200 *g*, 4°C). One milliliter of citrate buffer (pH 3) was added to 1 ml of the supernatant in a 20-ml culture tube with PTFE-lined caps (Prolabo, Paris, France) and extracted twice with 10 ml of ethyl acetate in a rotary shaker (Cenco, Breda, The Netherlands) for 15 min. The organic phase was transferred to a clean evaporating tube with Pasteur pipet. Ethyl acetate was evaporated at 40°C under a nitrogen stream. The residue was taken up with 100 μl of the mobile phase and 50-μl aliquots were injected into the system.

Plasma standard curves were prepared from a solution of indomethacin (10 μg/ml, prepared daily from a stock solution of 1 mg/ml in methanol and stored at 4°C in darkness up to one month) by serial dilutions with drug-free human plasma (0.02—1 μg/ml).

Calculations

Concentrations of indomethacin were determined from standard curves of peak height versus concentration. Linear regression analysis and interpolation were performed with a microcalculator (HP 97, Hewlett-Packard, Palo Alto, CA, U.S.A.).

Pharmacokinetic data and statistical analyses were performed with a 48 K Apple II computer (Apple Inc., Cupertino, CA, U.S.A.) using an interactive graphic package for pharmacokinetic analysis with a feathering method (LEFERCALC) [15].

RESULTS

Statistical validation of the method

The linearity of the method was evaluated in plasma in the concentration range of 0.02—1 μg/ml. The daily standard curve was obtained using the analytical procedure. The data are best described by a linear equation $Y = 1.076\ X - 0.003$ where X is concentration of indomethacin in μg/ml, and Y is peak height ratio of indomethacin to phenylbutazone. A mean correlation coefficient of 0.9963 ± 0.0028 was obtained, indicating a high degree of linearity (n=10) ($p < 0.001$).

The recovery of indomethacin from plasma was determined by comparing the ratio of indomethacin to phenylbutazone (internal standard) peak heights

in spiked plasma specimens (indomethacin 1 μg/ml, phenylbutazone 1 μg/ml), to the ratio in spiked plasma with only 1 μg/ml of phenylbutazone (1 μg of indomethacin added just before injection) (Table I).

TABLE I

RECOVERY OF INDOMETHACIN FROM PLASMA AND REPRODUCIBILITY

Amount added to plasma = 1 μg/ml.

Experiment No.	$(I/P)^{*}_{ref}$	$(I/P)^{**}_{extr}$	Yield (%)
1	0.946	0.792	83.7
2	0.864	0.706	81.7
3	0.970	0.741	76.4
4	0.867	0.789	91.0
5	0.930	0.815	87.6
6	0.985	0.821	83.4
7	0.904	0.704	77.9
8	0.988	0.791	80.1
9	0.940	0.725	77.1
10	0.976	0.821	84.1
Mean	0.937	0.768	82.3
S.D.	± 0.046	± 0.049	± 4.7
C.V. (%)	4.3	6.4	5.7

*$(I/P)_{ref}$ = peak height ratio of indomethacin to phenylbutazone; phenylbutazone added just before injection.

**$(I/P)_{extr}$ = peak height ratio of indomethacin to phenylbutazone after complete extraction.

Recovery (mean ± S.D.) was 82.3 ± 5.7% (n=10). Reproducibility was calculated in ten calibration curves in the range 0.02, 0.05, 0.1, 0.25, 0.5 μg/ml. The statistical analysis of indomethacin peak height versus phenylbutazone peak height gave correct results (C.V. = 6.4% at 1 μg/ml, about 20% at very low concentration 0.05 μg/ml) (Table II).

In the present analytical conditions, the minimum concentration that could be accurately measured was about 20 ng/ml (signal-to-noise ratio = 5) with a 1-ml plasma sample. Higher sensitivity (about 10 ng/ml) may be possible by increasing the plasma volume.

A chromatogram of blank and patient plasma 3 h after administration (volunteer who had received 75 mg of indomethacin) is shown in Fig. 1. The indomethacin concentration is about 1.5 μg/ml (plasma sample 1 ml, a.u.f.s. 0.16).

A major metabolite of indomethacin, deschlorobenzoyl indomethacin, is well separated from indomethacin and phenylbutazone (retention time = 5.5 min for indomethacin and 3.5 min for the deschlorobenzoyl metabolite).

Table III presents those drugs which were tested and found not to interfere with these assays (10 μg/ml of each). This does not rule out the possibility that metabolites of these drugs may interfere with these assays.

TABLE II

REPRODUCIBILITY OF INDOMETHACIN DETERMINATION IN PLASMA

Peak height ratio indomethacin/phenylbutazone (internal standard) = 0.5 μg/ml.

	Concentration (ng/ml)					Correlation coefficient
	20	50	100	250	500	
	0.1243	0.2142	0.4712	1.0148	2.0133	0.9923
	0.1212	0.2535	0.4870	1.135	2.145	0.9988
	0.0724	0.1960	0.5040	0.984	2.015	0.9971
	0.1556	0.3251	0.6934	1.274	2.348	0.9987
	0.1023	0.1866	0.6193	1.368	2.388	0.9947
	0.0686	0.2264	0.4198	0.944	2.1533	0.9979
	0.110	0.325	0.666	1.118	2.0906	0.9975
	0.125	0.235	0.561	0.9679	1.810	0.9981
	0.152	0.2235	0.4148	0.9310	2.407	0.9906
	0.0843	0.220	0.622	1.053	2.0988	0.9978
Mean	0.1116	0.2406	0.5459	1.0789	2.1458	0.9963
± S.D.	0.0322	0.04828	0.1008	0.1463	0.1887	0.0028
C.V. (%)	28.8	20.1	18.3	13.6	8.8	

In vivo study

The plasma concentration kinetics of indomethacin (mean of the ten volunteers) are shown in Fig. 2. A summary of the pharmacokinetic parameters is shown in Table IV (see Discussion).

DISCUSSION

Method

The choice of 250 nm for measurement of indomethacin was based on the UV spectra in the mobile phase.

Deproteinization of plasma is essential. The use of perchloric acid (0.66 *N*) gave poor recoveries. This is probably due to the higher degree of indomethacin adsorption to plasma proteins in an aqueous perchloric acid solution at pH <1 [16].

The use of organic solvents gave greater recovery. After trials, it was found that indomethacin recovery could be improved when the plasma was deproteinized with an equal volume of acetonitrile.

The acidic nature of indomethacin required extraction at low pH and the use of an acidic mobile phase to reduce band tailing by ion suppression. Citrate buffer at pH 3 was found to be the best for extraction. Diethyl ether and chloroform gave a coextracted lipid peak at $k = 21.2$ (20 min retention time). With less polar solvents (benzene, hexane), very low recoveries were obtained (<5%). Ethyl acetate resulted in the best quantitative extraction. The use of an appropriately acidic mobile phase renders the indomethacin molecule in an ionized lipophilic state, leading to retention on octadecylsilane columns.

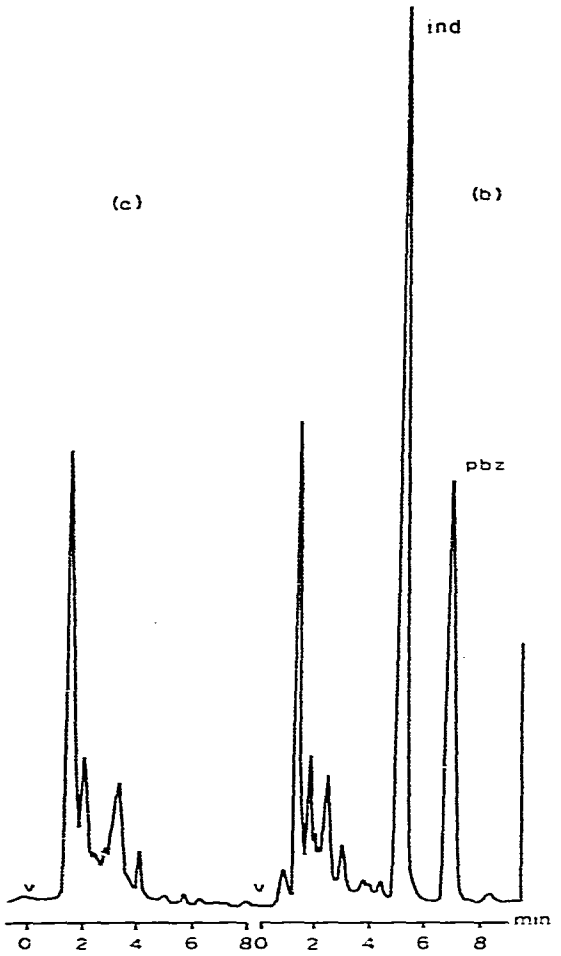

Fig. 1. (a) Chromatogram of a human blank plasma sample. (b) Chromatogram of a human volunteer plasma sample, collected 3 h after a single dose of 75 mg of Indocid®. Internal standard (phenylbutazone, pbz) = 1 μg/ml; calculated indomethacin (ind) concentration = 1.5 μg/ml.

TABLE III

SUBSTANCES CHECKED FOR INTERFERENCE

Quinine	Oxazepam
Quinidine	Clobazam
Phenobarbital	Imipramine
Secobarbital	Chlorimipramine
Meprobamate	Theophylline
Chloroquine	Salicylic acid
Promethazine	Acepromazine
Diazepam	Caffeine

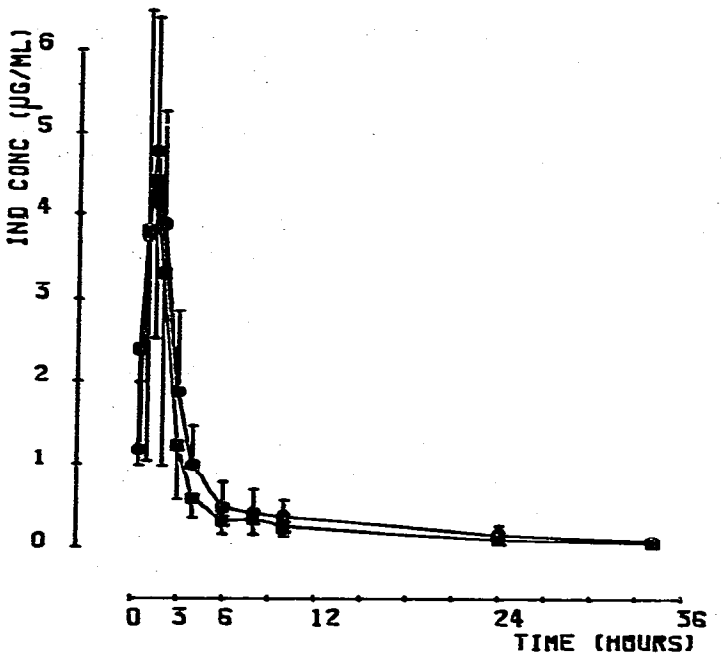

Fig. 2. Mean plasma concentration of indomethacin after a single oral dose (75 mg) (mean ± S.D., n=10). (□) Indocid®; (○) generic indomethacin.

TABLE IV

PHARMACOKINETIC PARAMETERS

Indomethacin given as a single oral dose (75 mg) in two formulations. Values represent mean ± S.D. for ten determinations for each formulation.

Parameters*	Indocid®	Generic indomethacin	Significativity**
t_{max} (h)	1.24 ± 0.27	1.24 ± 0.19	NS
C_{max} (µg/ml)	5.61 ± 2.98	5.48 ± 1.60	NS
$AUC_{0\to\infty}$ (µg ml^{-1} h^{-1})	15.4 ± 5.10	19.47 ± 8.33	NS
α (h^{-1})	1.32 ± 0.44	1.00 ± 0.18	NS
β (h^{-1})	0.058 ± 0.025	0.057 ± 0.0020	NS
$t_{½(\beta)}$ (h)	15.2 ± 7.3	14.0 ± 6.5	NS
A (µg/ml)	27.9 ± 2.2	25.8 ± 1.5	NS
B (µg/ml)	0.39 ± 0.03	0.62 ± 1.5	NS

*Pharmacokinetic symbols: t_{max} = time of the maximum concentration. C_{max} = maximum concentration in plasma. $AUC_{0\to\infty}$ = area under the plasma level curve extrapolated to infinity using Wagner equation: $AUC_{0\to\infty} = AUC_{0\to t} + \frac{C_t}{\beta}$, where C_t = plasma concentration at time t. A, B = coefficients of the two exponential functions. α = rate constant of distribution phase. β = rate constant of elimination phase. $t_{1/2(\beta)}$ = elimination half-life. $t_{1/2(\alpha)}$ = distribution half-life.

**Analysis of variance, t-test for paired data, Westlake, Wilcoxon and Duncan tests.

The p*K*a of indomethacin is 4.5 and a mobile phase of acetonitrile—water containing acetic acid (60 : 40) with an ionic strength of 0.1 (pH 3.8) was found to be suitable.

The solvent polarity parameter P' (Rohrschneider's parameter [17]) of this mixture was about 7.56 and the solvent strength parameter for the reversed phase (Snyder's parameter [18]) was 1.24.

Under these conditions, the capacity factors k of indomethacin and phenylbutazone were 3.7 and 4.7. The selectivity factor was 1.27 and the number of plates per meter for indomethacin was 15,525.

In vivo study

The pharmacokinetics of indomethacin in humans are only partly known and some findings appear contradictory. It is usually absorbed rapidly from the gut and a proportion may undergo enterohepatic recirculation [1, 19]. Holt and Hawkins [20] found complete elimination from the human body in 5 h, while Palmer et al. [21] could detect the drug in plasma for 32 h after intake. For several authors, the disappearance of indomethacin from plasma appears to consist of a fast primary phase ($t_{1/2}$ about 90 min) and a slower secondary elimination phase. The plasma elimination half-life in the beta phase ranges from 2.6 to 11.2 h [1].

The present work also investigated the disappearance of indomethacin from the plasma of ten subjects. With two formulations (Indocid® and generic form), a single oral dose (75 mg) give a rapid increase in the plasma concentration (t_{max} = 1.24 ± 0.22 h, C_{max} = 5.54 ± 2.3 μg/ml) but it was impossible to distinguish clearly between a true distribution phase and an elimination phase for these decreases.

An initial plasma half-life of 1.32 ± 0.44 h^{-1} was found, which is in good agreement with other findings (about 90 min [1, 22]).

The change in the elimination rate following these initial phases can be explained by enterohepatic recirculation and/or binding to proteins or tissues, from which the drug is slowly released.

A half-life of 13.6 ± 6.9 h^{-1} was found (n=20) for these phases. The plasma half-life in the terminal exponential phase was much longer than previously reported by Alvan et al. [1] for a similar dose. An analysis of variance and Student's test were applied to this parameter between the present work and the data of Alvan et al. (Table V). There are highly significant differences between the two groups (p <0.001). Considerable intra- and interindividual variations of indomethacin pharmacokinetics may perhaps explain these differences. Work is in progress to confirm and explain this higher value for the half-life of the beta phase.

CONCLUSION

A reversed-phase HPLC assay is described for the measurement of plasma indomethacin with sufficient sensitivity to measure the drug in pharmacokinetic studies.

The results indicate that the plasma curve of indomethacin is biexponential with an initial rapid phase (half-life of 1.32 ± 0.44 h^{-1}) followed by a complex

TABLE V

PLASMA HALF-LIVES (BETA PHASE)

Values are expressed in h^{-1}.

Subject	Indomethacin (generic)	Indocid®
CHA	20.0	15.2
COU	9.5	8.1
EMG	10.5	15.2
GAZ	30.3	32.3
LAL	11.1	10.1
LAM	14.3	10.3
LEC	10.5	11.2
LOM	8.5	7.1
MOR	16.3	15.3
PET	7.5	8.6
Mean ± S.D.	14.0 ± 6.5	15.2 ± 7.3
Significance (analysis of variance, *t*-test)	NS	($p < 0.001$)

	Indocid®* (n=10)	Alvan et al. [1] (n=13)	Indomethacin* (generic) (n=10)
$t_{1/2}$ indomethacin (h^{-1})	15.2 ± 7.3	5.9 ± 1.6	14.0 ± 6.5
Significativity	$p < 0.001$		$p < 0.001$

*Present work.

slower beta phase with a half-life of 13.6 ± 6.9 h^{-1} in ten subjects and two formulations.

There is no evidence for statistical differences between these two galenic forms (analysis of variance, Student's test for paired data, Wilcoxon and Duncan test).

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REFERENCES

1 G. Alvan, M. Orme, L. Bertilsson, R. Ekstand and L. Palmer, Clin. Pharmacol. Ther., 18 (1975) 365–373.
2 M.J. van der Meer and H.K.L. Hundt, J. Chromatogr., 181 (1980) 282–285.

3 R.G. Sibeon, J.D. Baty, N. Baber, K. Chan and M.L'E. Orme, J. Chromatogr., 153 (1978) 189—194.
4 D.G. Ferry, D.M. Ferry, P.W. Moller and E.G. McQueen, J. Chromatogr., 89 (1974) 110—112.
5 A. Arbin, J. Chromatogr., 144 (1977) 85—92.
6 L.E. Hare, C.A. Ditzles and D.E. Duggan, J. Pharm. Sci., 66 (1977) 486—489.
7 H.B.A. Hucker, A.G. Zacchei, S.V. Cox, D.A. Brodie and M.H.R. Cantwell, J. Pharmacol. Exp. Ther., 153 (1966) 237—249.
8 E. Hvidberg, H. Hansen en J.A. Jansen, Eur. J. Clin. Pharmacol., 4 (1972) 119—224.
9 G.G. Skellern and E.G. Salole, J. Chromatogr., 114 (1975) 483—485.
10 E. Lin and L.Z. Benet, in W. Sadee and G.C.M. Beilen (Editors), Drug Level Monitoring, Wiley, New York, 1980, p. 281.
11 C.P. Terwey-Groen, S. Heemstra and J.C. Kraak, J. Chromatogr., 181 (1980) 385—397.
12 J.L. Schimek, N.G.S. Rao and S.K. Wahba-Khalil, J. Liquid Chromatogr., 4 (1981) 1987—2013.
13 S.J. Soldin and T. Gero, Clin. Chem., 25 (1979) 589—591.
14 J.J. Kirkland, Chromatographia, 8 (1975) 661.
15 G. Fredj and A. Astier, personnel program.
16 M. Otagnini and J.H. Perrin, Biochem. Pharmacol., 26 (1977) 283.
17 L. Rohrschneider, Anal. Chem., 45 (1973) 242.
18 L.R. Snyder, J. Chromatogr., 92 (1974) 223.
19 K.C. Kwan, G.O. Breault, E.R. Umbenhauer, F.G. McMahon and D.E. Duggan, J. Pharmacokin. Biopharm., 4 (1976) 225—280.
20 L.P.J. Holt and C.F. Hawkins, Brit. Med. J., i (1965) 1354—1356.
21 L. Palmer, L. Bertilsson, G. Alvan, M. Orme, G. Sjoqvist and B. Holmstedt, in H.J. Robinson and J.R. Vane (Editors), Prostaglandin Synthetase Inhibitors, Raven Press, New York, 1974, pp. 91—97.
22 D.E. Duggan, A.F. Hogans, K.C. Kwan and F.G. McMahon, J. Pharmacol. Exp. Ther., 181 (1972) 573—575.