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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF INDOMETHACIN AND ITS APPLICATION IN PHARMACOKINETICS IN HEALTHY VOLUNTEERS

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SUMMARY

A new sensitive high-performance liquid chromatographic method for indomethacin from plasma on a reversed-phase column (C_{18}) has been developed. The method involves precipitation of plasma with perchloric acid followed by diethyl ether extraction. The assay is quantitative down to 0.25 $\mu g\ ml^{-1}$ from a 200-μl aliquot of plasma with a detection limit of 0.1 $\mu g\ ml^{-1}$ and a recovery of approximately 90%.

The method was applied to single-dose studies with volunteers under various dietary restrictions. The results of these studies indicated that intrasubject variability within these regimens may be as important a factor as the intersubject variability already documented for this drug. These results have important implications in the determination of bioavailability and pharmacokinetic parameters of this drug.

INTRODUCTION

Indomethacin has been quantitatively determined in biological fluids by means of gas chromatography (GC) [1—5] using an electron-capture detector. These methods, however, require some derivatization technique which in the case of reaction with diazomethane converts a percent of any O-desmethyl-indomethacin present back to the parent compound. In addition many of the GC methods lack an internal standard or utilize an internal standard not readily available.

Thin-layer chromatographic methods [6, 7] reported may be too cumbersome and time consuming when large numbers of plasma samples are to be analyzed.

Radioisotope dilution techniques exist but require administration of radioactivity to patients [8—10] or lack specificity. The radioimmunoassay technique of Hare et al. [11] is sensitive but indomethacin glucuronide is highly

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cross reactive. The combined GC—mass fragmentographic techniques [12, 13] are sensitive but also require derivatization.

The use of reversed-phase high-performance liquid chromatography (HPLC) in the analysis of plasma levels of indomethacin has been advocated [14—16]. The procedure of Skellern and Salole [16], requiring rather large plasma samples (1 ml), provides no precision data for proper evaluation. Soldin and Gero's method [15] is based on ethylene dichloride extraction of plasma which results in 66—68% recovery of the drug. However, the coefficient of variation at 463 and 927 ng/ml was rather large (> 10%). The described procedure is simple and sensitive, requires no derivatization technique, and metabolite or concomitantly administered drugs such as salicylic acid do not interfere with the assay. This method uses the protein precipitation technique of Terweij-Groen et al. [14], with the modification of a more convenient diethyl ether extraction solvent over methylene chloride, and quantitative recovery of the drug (88—90%). The application of this procedure to single-dose pharmacokinetic studies in healthy volunteers clearly establishes the large intrasubject variability which must be seriously taken into account in future bioavailability studies.

EXPERIMENTAL

Materials

Indomethacin and mefenamic acid were generously donated by ICN Laboratories, Montreal, Canada. Diethyl ether was distilled prior to use, all chromatographic solvents were HPLC grade, and all other chemicals were analytical reagent grade.

Apparatus

A Waters Model M-45 liquid chromatographic pump (Waters Scientific, Mississauga, Canada) and a Model 7125 Rheodyne valve-loop injector fitted with a 500-μl loop were employed (Technical Marketing Associates, Calgary, Canada). An Ultrasphere ODS 250 × 4.6 mm I.D. column (particle size 5 μm) (Beckman Instruments, Toronto, Canada) was connected to a Waters Model 440 fixed-wavelength detector set at 254 nm (Waters Scientific). The mobile phase was 0.1 *M* acetic acid—acetonitrile (30:70) pumped at a flow-rate of 1 ml min^{-1}. The solvent mobile phase was degaaased by refluxing for 5 min transferred to the solvent reservoir. All chromatography was carried out at ambient temperature.

Internal standard

A stock solution of mefenamic acid of 200 $\mu g\ ml^{-1}$ was made weekly in 50% aqueous ethanol and stored at 4°C.

Preparation of standard curve

A stock solution of indomethacin of 400 $\mu g\ ml^{-1}$ was made weekly in 50% aqueous ethanol as previously described [1] and stored at 4°C. Serial dilutions in 50% aqueous ethanol were made daily so that 20 μl of solution would correspond to a concentration range of 8.00—0.25 $\mu g\ ml^{-1}$ of plasma.

Extraction of samples

To a 10-ml PTFE-lined screw-capped test tube were added 200 μl of plasma and 20 μl of internal standard solution containing 4 μg of mefenamic acid. The sample was mixed for 20 sec (Vortex Genie, Fisher Scientific, Edmonton, Canada) and 1 ml of 0.3 *M* perchloric acid added. The sample was remixed for 5 min (Evapomix, Fisher Scientific); then 6 ml of freshly distilled diethyl ether was added. The tube was tightly capped and remixed as above for 10 min. After centrifugation for 5 min at room temperature at 1725 *g* (TJ6 centrifuge, Beckman Instruments) the ether layer was transferred to another screw-capped tube containing 0.5 ml of 0.2 *M* phosphate buffer pH 7.2. The sample was capped tightly and mixed for 10 min and centrifuged for 5 min as before. The upper organic layer was transferred to another screw-capped test tube containing anti-bumping granules (BDH Chemicals, Toronto, Canada) and evaporated to dryness at 55°C (Thermolyne Dri-Bath, Fisher Scientific). The tube was allowed to reach room temperature after which 500 μl of mobile phase were added and the sample was mixed for 30 sec. An aliquot of 100—200 μl was injected into the HPLC instrument.

Plasma level study

Three healthy male volunteers weighing 61 kg, 80 kg, and 55 kg, respectively, were fasted overnight and received 50 mg of indomethacin (two 25-mg tablets), with either water (250 ml) or milk (250 ml). On a third occasion after a standardized normal breakfast these same volunteers received 50 mg indomethacin with water (250 ml).

The three treatments were each given twice each in turn. During the first week indomethacin was administered with water after fasting overnight. The time between dosages was three days. During the second week indomethacin was given with milk after an overnight fast. During the third week indomethacin with water, following a normal breakfast, was administered. Blood samples (10 ml) were obtained by venipuncture and collected in heparinized tubes (Vacutainers, Becton and Dickinson, Mississauga, Canada) at 0, 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.5, 6.0, and 8.0 h following each dose. The blood samples were not permitted to touch the rubber stoppers upon collection and the plasma was removed and either analyzed immediately or stored at −20°C until just prior to analysis.

Recovery study

For the determination of recovery five replicate samples at levels of 1 and 4 μg ml^{-1} for indomethacin, and 1 μg ml^{-1} for mefenamic acid, were spiked in fresh blank heparinized plasma and run through the procedure as described for extraction of samples. The absolute peak heights obtained for the extracted samples were compared with those of fresh standards of indomethacin and mefenamic acid in mobile phase.

Quantitation

Standard curves for indomethacin were constructed by chromatographing spiked plasma extracts and plotting the peak height ratios obtained for the drug to the internal standard versus the concentration of the drug. Samples from volunteers were analyzed at the same time as calibration standards.

RESULTS AND DISCUSSION

Indomethacin and the internal standard gave sharp symmetrical peaks under the described conditions given in Experimental with retention times of 4.8 and 8.10 min, respectively (peaks a and b in Fig. 1B and C).

Fig. 1A shows a chromatogram of a 200-μl extract of fresh blank plasma which was processed as described in Experimental. Fig. 1B shows a chromatogram, obtained when the method was applied to spiked plasma containing 4 μg ml^{-1} of indomethacin and 20 μg ml^{-1} of the internal standard mefenamic acid, where it is evident that no endogenous peaks interfere. Fig. 1C is a chromatogram of a 200-μl plasma sample 7 h post dose from a volunteer (80 kg) who received two 25-mg tablets of a commercial formulation of indomethacin. The sample was estimated to contain 0.31 μg ml^{-1} of indomethacin.

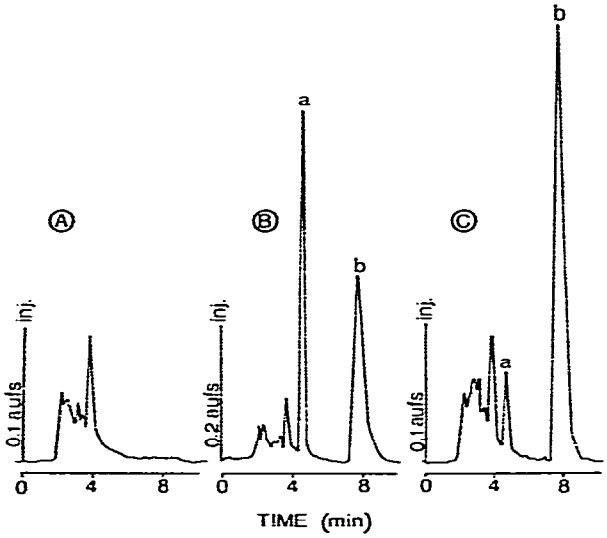

Fig. 1. Chromatograms of extracts from 200 μl of plasma. A, Blank plasma; B, plasma spiked with indomethacin (a, 4 μg ml^{-1}) and internal standard mefenamic acid (b, 20 μg ml^{-1}); C, plasma sample from a volunteer 7 h post dose (two 25-mg tablets) estimated to contain 0.31 μg indomethacin per ml of plasma.

The accuracy and precision of the assay is demonstrated in Table I, the results of which are based on six determinations for each concentration of indomethacin ranging from 0.25 to 8.00 μg ml^{-1} of plasma. The calibration curve obtained was linear from 0.25 to 8.00 μg ml^{-1} ($y = mx + b$) with a mean slope value of $m = 2.58$, an intercept of $b = 0.06$, and $r^2 = 0.99$.

Application of this method is shown in Fig. 2 for plasma concentrations over 8 h in a fasted volunteer (80 kg) who received 2 × 25 mg of indomethacin with water.

For confirmation that there was no metabolic interference under the peak due to indomethacin, pooled plasma extracts from volunteers were combined and injected into the HPLC instrument. The resulting effluent was collected at the retention time for indomethacin, the mobile phase was evaporated un-

TABLE I

ESTIMATION OF INDOMETHACIN ADDED TO PLASMA BY HPLC

$n = 6$.

Indomethacin added ($\mu g\ ml^{-1}$)	Mean peak height ratio drug/I.S.	S.D.	C.V. (%)
0.25	0.099	0.003	3.05
0.50	0.182	0.007	3.85
1.00	0.370	0.012	3.24
2.00	0.712	0.016	2.25
4.00	1.513	0.053	3.50
8.00	3.082	0.097	3.15

Mean C.V. = 3.17%

$y = 2.58x + 0.06$ ($r^2 = 0.99$)

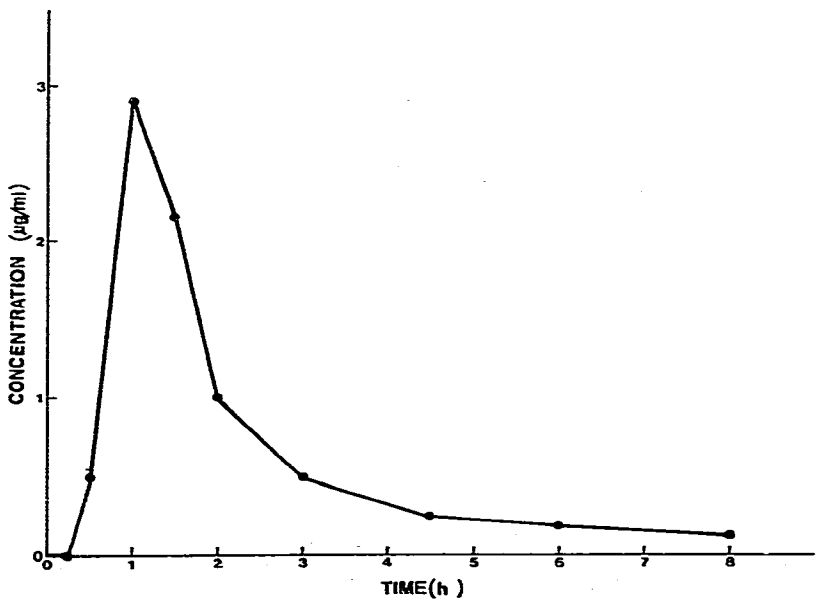

Fig. 2. Plasma concentration versus time profile for a volunteer (80 kg) who received 2 × 25 mg indomethacin with water after fasting overnight.

der vacuo at 30°C to dryness, and the residue dissolved in methanol evaporated and run by solid probe using a VG Micromass MM16F MS System (VG Micromass, Altrincham, Great Britain). The mass spectrum indicated the presence of only indomethacin.

The recovery of indomethacin and the internal standard are shown in Table II and are in the order of 89.43 ± 0.98% and 61.37 ± 0.82%, respectively.

The effect of various treatments of oral indomethacin is shown in Table III where each subject's AUC_0^8 is summarized. It is evident that there is not only

TABLE II

RECOVERY OF INDOMETHACIN AND MEFENAMIC ACID FROM PLASMA

Drug	Amount added to 200 μl plasma (μg)	Amount recovered (μg)	Mean recovery (%)	S.D. of percent recovery
Indomethacin	1	0.88	88.42	0.47
	4	3.62	90.44	1.48
			Mean 89.43 ± 0.98	
Mefenamic acid	4	2.45	61.37	0.82
			Mean 61.37 ± 0.82	

TABLE III

AUC_0^8 (μg h ml^{-1}) OF THREE VOLUNTEERS DOSED WITH INDOMETHACIN UNDER THREE DIFFERENT TREATMENTS

A = first dose; B = second dose.

Volunteer	Treatment					
	Water		Milk		Breakfast	
	A	B	A	B	A	B
1 (61 kg)	4.82	4.22	3.88	5.67	3.77	2.97
2 (80 kg)	4.85	6.03	6.07	5.18	4.54	6.36
3 (55 kg)	10.68	6.09	9.52	7.63	7.24	5.61

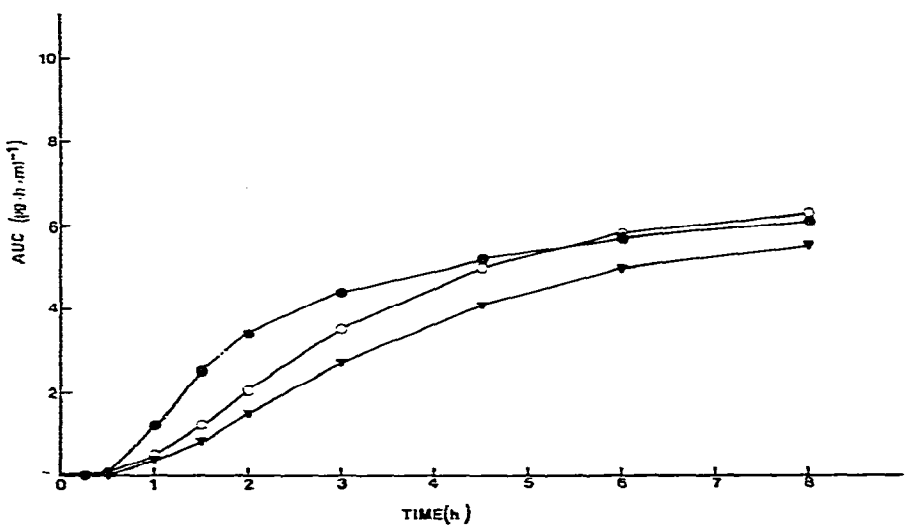

Fig. 3. Truncated mean AUC versus time profiles for three volunteers each dosed twice with indomethacin under three different conditions: overnight fast, drug administered with water (●); overnight fast, drug administered with milk (○); normal breakfast, drug administered with water (▲).

intersubject variation but also intrasubject variation. Volunteers 2 and 3 show the greatest variation, in volunteer 2 occurring with breakfast (ratio dose B/A of 140%) and in volunteer 3 occurring with water only (ratio dose B/A of 57%). Volunteer 2 exhibited the least variation with milk but the two treatments still had a B/A ratio of 85%. It can be seen that there is no observed intrasubject trend towards treatment and that, even when experimental and sampling error is accounted for, these intrasubject differences still remain. The truncated mean AUC time curves from these data are presented in Fig. 3. By plotting truncated areas under the blood level curve against time one is able to clearly observe simultaneously both the rate and extent of drug disposition. In Fig. 3 it is clear that administration to fasting subjects with water provides higher plasma levels with a shorter t_{max} as compared to the other two treatments. In addition the total bioavailability after the eight hours is practically equal for all three treatments. These observations are in agreement with those presented by Wallusch et al. [17]. In the latter study, however, each dose was given only once in each subject under each regimen, hence any intrasubject variance would not be evident. The mean values obtained, however, do simulate those findings.

In conclusion a new simple HPLC method for indomethacin has been developed and has been demonstrated to be applicable to single-dose pharmacokinetic studies of this drug in man. Our experimental results suggest that not only intersubject variability but also intrasubject variability exists for this drug. This intrasubject variability should be further investigated in a large population and must be considered in future pharmacokinetic and bioavailability studies of this drug.

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