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RAPID DETERMINATION OF INDOMETHACIN AND SALICYLIC ACID IN SERUM BY MEANS OF REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the quantitative analysis of indomethacin and salicylic acid in blood serum and urine by high-performance liquid chromatography is described. A C_{18}-bonded silica was used as the stationary phase and mixtures of ethanol, *n*-butanol and aqueous buffer as the mobile phase. Before injection the serum is deproteinized and extracted in one step.

The recovery of the extraction was found to be 88% and 77% for indomethacin and salicylic acid, respectively. The relative standard deviations of the analysis for 0.5 μg indomethacin and 5 μg salicylic acid per ml serum were 3.6% and 3.2%, respectively. The detection limits for indomethacin and salicylic acid were 2 ng. This corresponds for both substances to 0.1 μg/ml serum for an injection volume of 100 μl.

The method enables simultaneous determination of possibly formed metabolites. A number of concurrently administered drugs do not interfere with the analysis. The interactive effects of co-medication of indomethacin and salicylic acid on the serum concentration of indomethacin is demonstrated by measuring the pharmacokinetic curves.

INTRODUCTION

Indomethacin [1-(*p*-chlorobenzoyl)-5-methoxy-2-methyl-indole-3-acetic acid], an anti-inflammatory drug, is frequently used in combination with salicylate in the treatment of rheumatoid arthritis [1]. It is well known that indomethacin rapidly metabolizes in the body and that some of the metabolites lack anti-inflammatory activity [2—8]. However, conflicting results have appeared in the literature about the influence of salicylates on the plasma level and metabolism of indomethacin [9—14].

In order to investigate this effect, a rapid and selective method for the simultaneous determination of indomethacin, its metabolites and salicylic acid in serum is required. Until now, extraction procedures combined with radioactive [3—5, 10] or fluorimetric [11—13] techniques have been applied.

Although these methods are quite sensitive, they do not discriminate between the drug, its metabolites [3, 10—13] and, in the case of fluorimetry, salicylate [15] and other administered drugs [3].

A number of chromatographic methods has proved to be successful [2, 6, 14, 16—19]. In order to determine indomethacin by means of gas chromatography (GC), derivatization is required [6, 14, 16—18]. This step introduces additional uncertainties in the quantitative analysis. Anion-exchange chromatography has been used for the separation of indomethacin, its metabolites and salicylic acid prior to radioactive quantitation [7—9]. However, the limit of determination of this method did not meet the requirements for pharmacokinetic studies. Recently, thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) procedures for the analysis of indomethacin have been reported [19—21]. However, these methods do not allow the simultaneous determination of metabolites and salicylic acid.

In the present paper we describe a method for the quantitative determination of indomethacin, its major metabolites and salicylic acid by means of HPLC with UV detection. The method has been successfully applied to a pharmacokinetic study of the influence of salicylate on the plasma level and metabolism of indomethacin.

EXPERIMENTAL

Apparatus

The HPLC experiments were carried out on a high-pressure liquid chromatograph (Siemens SP 200, Siemens, Karlsruhe, G.F.R.) using UV detection at 235 nm (Pye-Unicam LC-UV, Philips, Eindhoven, The Netherlands), a high-pressure sampling valve (Valco CV-6-UHP a, Valco Instruments, Houston, Texas, U.S.A.) with a 100-μl loop, a stainless-steel column (250 mm × 4.6 mm I.D.) and a linear potentiometric recorder (Servogor 542, Goerz, Vienna, Austria).

The acidic medium requires that all connections are made of stainless-steel 316 capillary tubing and stainless-steel Swagelok couplings. For the extractions a Vortex mixer (Scientific Industries, Springfield, N.Y., U.S.A.) was used. The experiments were carried out at room temperature.

Materials

In all experiments double-distilled water was used. A commercially available HPLC column containing an alkyl-modified material (Zorbax ODS, 5 μm, Dupont Instruments, Newton, Conn., U.S.A.) was used. All chemicals were of analytical reagent grade.

Procedures

The HPLC column was washed successively with 75% (w/v) ethanol and the appropriate eluent (both 100 times the column volume). Blood samples were drawn by venipuncture and centrifuged (10 min, 300 *g*). The blood serum was deep-frozen until assay.

For deproteinization, 0.3 ml serum was mixed with 1 ml 0.3 *M* perchloric acid of pH 0.7 in a glass-stoppered centrifuge tube (25 ml). After 10 min, 4 ml

dichloromethane was added and the aqueous phase was extracted by mixing 1 min on a vibration mixer. After centrifugation (15 min, 300 *g*), the aqueous phase was removed. A 3-ml portion of the organic phase was transferred to another centrifuge tube and evaporated until dryness under a stream of nitrogen.

The same extraction procedure was followed for urine starting with 1.3 ml urine (acidified to pH 1 with 70% (w/v) perchloric acid). In order to hydrolyze the glucuronides 0.65 ml urine (pH 5) was mixed with 0.63 ml 0.1 *M* acetate buffer (pH 5), containing 10^4 U β-glucuronidase per ml. After incubation at 37° during 1 h, 0.02 ml 70% (w/v) perchloric acid was added before extraction at pH 1.

The residues were dissolved in 1 ml of the eluent and centrifuged (1 min, 300 *g*). The supernatant was injected by means of a sample loop of 100 μl volume. To avoid memory effects the sample loop was rinsed with water after each injection. To prevent contamination the glassware used in contact with dichloromethane was rinsed with methanol and dried with nitrogen.

Standard solutions of indomethacin in 50% (w/v) ethanol were found to be stable for several weeks. In contradiction to earlier reports [17], in our experiments no loss of indomethacin and salicylic acid from frozen serum and urine samples was found during one month sample storage.

RESULTS AND DISCUSSION

In order to investigate the interactive effects of co-medication of indomethacin and salicylic acid, knowledge about their metabolic pathways is required. Fig. 1 shows which metablites and conjugates of indomethacin and salicylic acid have been found in serum and urine [2, 4, 22]. The conjugates mainly occur in urine [3, 5].

For the simultaneous determination of indomethacin, its main metabolites and salicylic acid in serum, the applicability of an alkyl-modified silica in combination with mixtures of alcohols and aqueous buffer was investigated.

In order to determine optimal chromatographic conditions the influence of the type and concentration of the organic modifier and pH on the retention of the compounds in question was investigated.

The results of these experiments showed that the logarithm of the capacity ratio of all compounds changes linearly with the concentration of *n*-propanol as is commonly observed in reversed-phase systems. However, it was found that the stability of the phase system decreased at increasing concentration of *n*-propanol.

For that reason also *n*-butanol was tested. When *n*-butanol was used as modifier at a concentration as large as possible with respect to solubility [6% (w/v) *n*-butanol], the capacity ratio of indomethacin was too large to measure. To increase the concentration of *n*-butanol, mixtures of *n*-butanol, ethanol and aqueous buffer were used as the eluent. Using these eluents a remarkable improvement of the column stability was observed. Moreover, the viscosity of the eluent was much lower than with *n*-propanol, as was noticed previously [23].

The dependence of the capacity ratios on the concentration of *n*-butanol at a

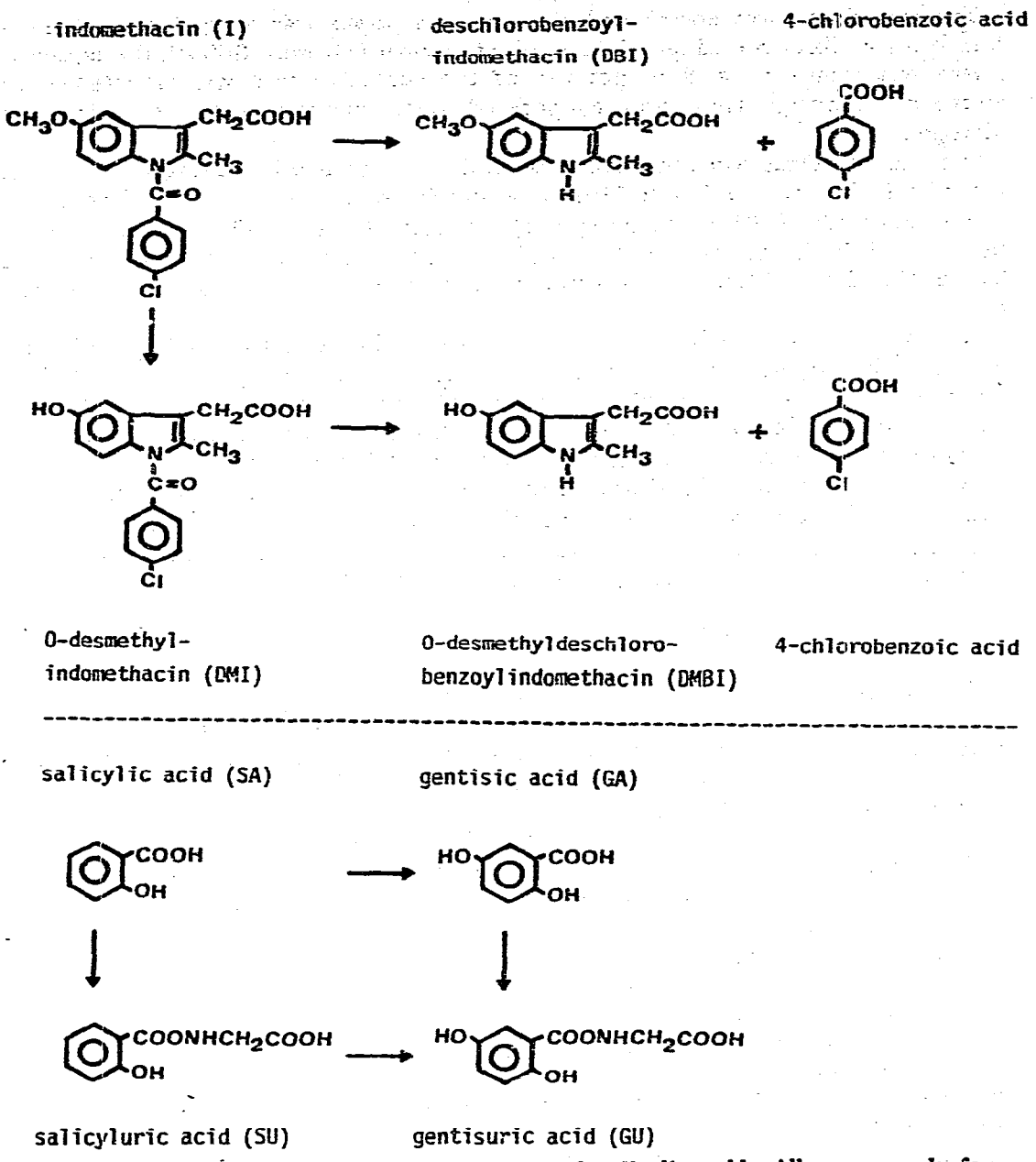

Fig. 1. Metabolic pathways of indomethacin and salicylic acid. All compounds form conjugates with glucuronic acid at the —COOH or the —OH group (RCOOH → $RCOOC_6H_9O_6$; R'OH → $R'OC_6H_9O_6$).

fixed concentration of 15% (w/v) ethanol is shown in Table I.

The plot of the logarithm of the capacity ratios versus the concentration of *n*-butanol now deviates from linearity. This is probably due to a change in the amount of ethanol, adsorbed at the column, with the concentration of *n*-butanol in the eluent (e.g. mixed adsorbed phase).

TABLE I

INFLUENCE OF THE CONCENTRATION OF *n*-BUTANOL IN THE ELUENT ON THE CAPACITY RATIO (k_i') AT A FIXED CONCENTRATION OF ETHANOL

The eluent consisted of 7—15% (w/v) *n*-butanol—15% (w/v) ethanol—0.08 *M* perchloric acid—0.05 *M* phosphate, pH 2.3.

Compound	k_i'			
	n-Butanol concentration (%, w/v)			
	7	8	10	15
2,5-Dihydroxybenzoic acid	0.80	0.77	0.68	0.61
5-Methoxy-2-methyl-indole-3-acetic acid	0.96	0.86	0.73	0.64
Salicylic acid	3.39	2.91	2.19	1.27
4-Chlorobenzoic acid	6.59	5.20	3.45	1.63
Indomethacin	12.30	11.12	6.11	2.08

In all further experiments the eluent consisted of mixtures of *n*-butanol, ethanol and aqueous buffer. For these eluents the effect of pH was investigated with respect to the separation of indomethacin, salicylic acid and their metabolites from drugs, which generally occur in aspirin tablet formulations. The results of these experiments are shown in Table II.

TABLE II

INFLUENCE OF pH ON THE CAPACITY RATIO (k_i')

The eluent consisted of 13% (w/v) *n*-butanol—13% (w/v) ethanol—0.08 *M* perchloric acid—0.05 *M* phosphate, pH 2—6.5.

Compound	k_i'			
	pH			
	2	3.5	5.0	6.5
5-Hydroxy-2-methyl-indole-3-acetic acid	0.61	0.58	0.27	0.02
4-Hydroxyacetanilide	0.64	0.71	0.72	0.70
Caffeine	0.85	0.93	0.93	0.90
2,5-Dihydroxybenzoic acid	1.21	0.86	0.22	0.15
Salicylamide	1.23	1.35	1.36	1.33
Acetylsalicylic acid	1.24	1.28	0.54	0.18
5-Methoxy-2-methyl-indole-3-acetic acid	1.60	1.52	1.04	0.20
Phenacetin	1.71	1.86	1.86	1.82
Salicylic acid	2.67	1.99	0.47	0.30
O-Desmethylindomethacin	3.18	3.35	2.30	0.88
4-Chlorobenzoic acid	3.52	3.65	2.00	0.60
Indomethacin	5.18	5.45	3.96	1.32

It can be seen that the separation between the relevant compounds is optimal in the pH range 2—4. In Fig. 2 the separation of test mixtures of a number of anti-inflammatory drugs is shown at different pH values. As shown in Fig. 3 salicylic acid is well resolved from indomethacin and its metabolites at pH 3.45.

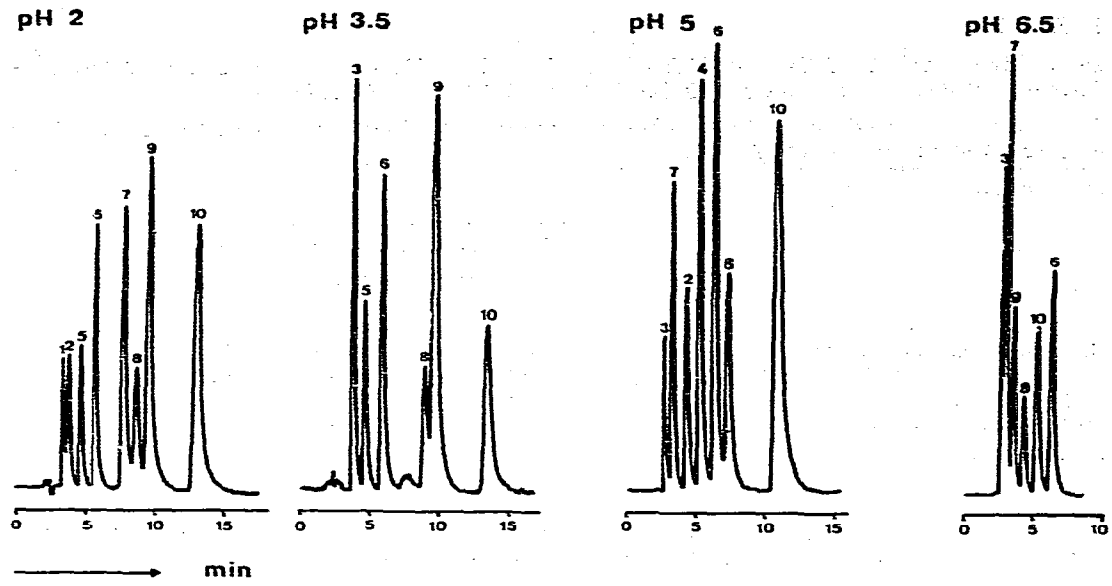

Fig. 2. The effect of pH on the separation of anti-inflammatory drugs. Eluent, 13% (w/v) *n*-butanol—13% (w/v) ethanol—0.08 *M* perchloric acid—0.05 *M* phosphate; pH 2—6.5, column, 250 mm × 4.6 mm I.D.; injection volume, 100 µl; wavelength 235 nm. Peaks: 1, 4-hydroxyacetanilide; 2, caffeine; 3, 2,5-dihydroxybenzoic acid; 4, salicylamide; 5, acetylsalicylic acid; 6, phenacetin; 7, salicylic acid; 8, O-desmethylindomethacin; 9, 4-chlorobenzoic acid; 10, indomethacin.

Quantitative analysis

Precision. The linearity of the chromatographic method was tested by injecting a volume of 100 µl of solutions of indomethacin (0.1—2.5 µg/ml) and salicylic acid (1—25 µg/ml). A correlation coefficient of 0.9999 was found for the linear relationship between peak height and amount of indomethacin and salicylic acid. The relative standard deviation for replicate analyses of indomethacin was 1.3% at 1 µg/ml ($n = 10$) and of salicylic acid 1.5% at 10 µg/ml ($n = 10$).

The detection limit, defined as three times the standard deviation of the noise (about $3 \cdot 10^{-5}$ a.u.) was determined to be 2 ng for both indomethacin and salicylic acid.

Recovery and reproducibility. Using a procedure from the literature [24], the deproteinized serum can be injected directly onto the column. However, compared with the recovery of salicylic acid (90%) for indomethacin a rather low recovery was found (50%). This must be attributed to the higher degree of adsorption of indomethacin to the serum proteins in an aqueous perchloric acid solution of pH < 1 [25, 26].

It was found that the recovery of indomethacin can be improved when the serum was deproteinized with perchloric acid and extracted with dichloromethane in one step, according to a method reported in the literature [27].

To determine the recovery and reproducibility of this method, known amounts of indomethacin and salicylic acid were added to blank serum before

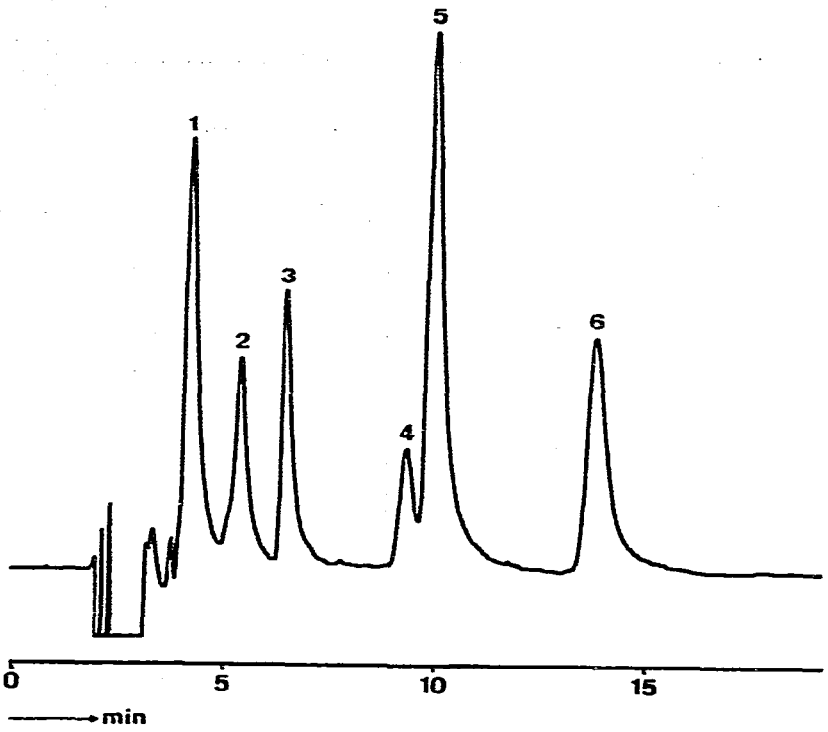

Fig. 3. The separation of a test mixture of salicylic acid, indomethacin and its metabolites. Eluent, 13% (w/v) *n*-butanol—13% (w/v) ethanol—0.08 *M* perchloric acid—0.05 *M* phosphate; pH 3.45; column, 250 mm × 4.6 mm I.D.; injection volume, 100 µl; wavelength, 235 nm; for abbreviations see Fig. 1. Peaks: 1, DMBI; 2, DBI; 3, SA; 4, DMI; 5, 4-chlorobenzoic acid; 6, indomethacin.

extraction. The extracts were analyzed by HPLC. The recovery from serum was found to be 87.5 ± 3.6% for indomethacin measured within the range 0.5—10 µg/ml serum and 76.6 ± 3.2% for salicylic acid within the range 5—100 µg/ml serum.

For aqeous solutions (acidified to pH 1 with perchloric acid) a recovery of 95.0 ± 2.0% was found for indomethacin and 80.0 ± 2.0% for salicylic acid. These results show that only a minor part of the compounds remained adsorbed at the serum proteins.

Pharmacokinetic study

The effect of concurrent administration of salicylic acid and indomethacin on their concentrations in serum was investigated by measuring a pharmacokinetic curve. Drugs, which generally occur in aspirin tablet formulations (see Table II), do not interfere with the analysis of indomethacin, salicylic acid and their metabolites at an eluent composition being selected on the basis of Tables I and II. A number of drugs commonly administered to rheumatic patients such as prednisone, glibenclamide, pentazocine and diclofenac do not interfere with the analysis.

Blood samples were taken from three patients (1 male, 2 female) with rheumatoid arthritis being treated with indomethacin and salicylic acid according to the medication scheme shown in Table III. Every fourth day of

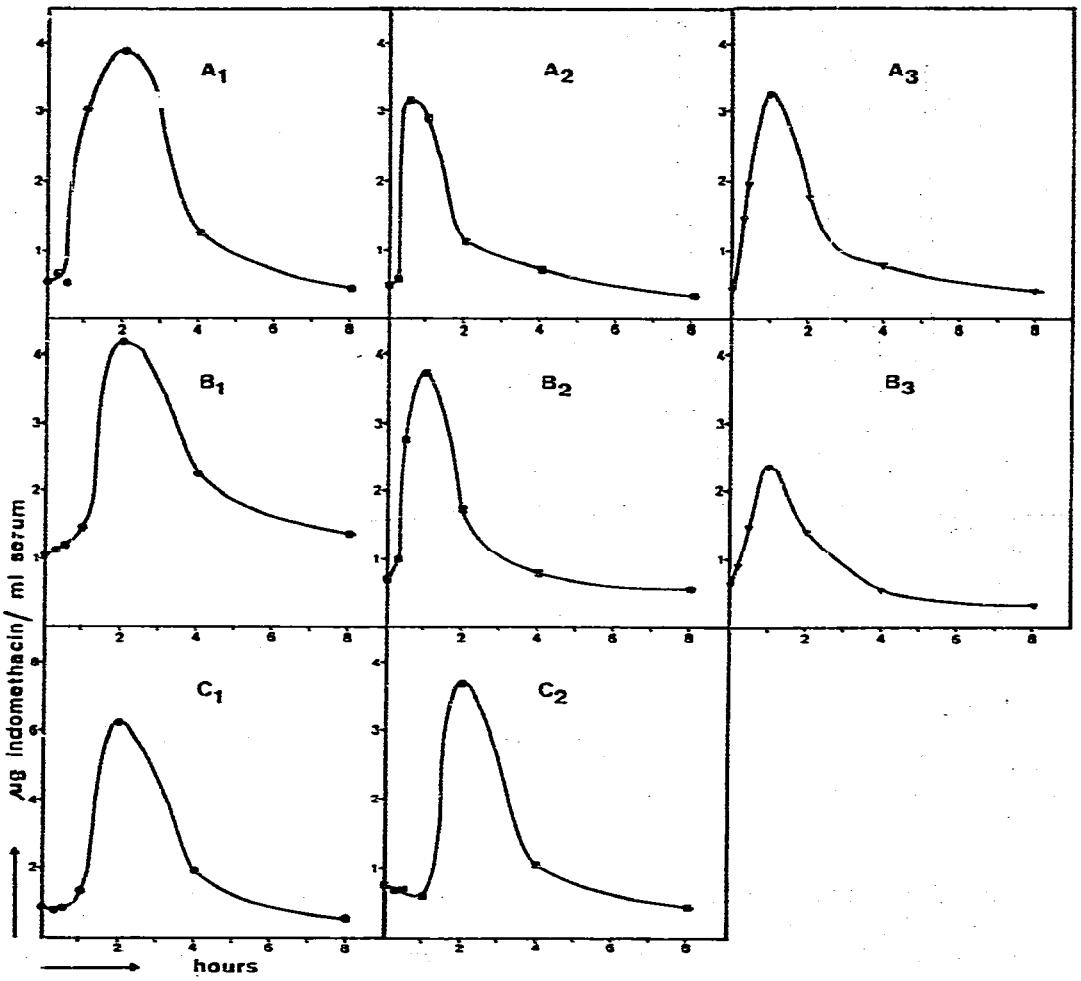

Fig. 4. Pharmacokinetic curves of indomethacin in the presence and in the absence of concurrently administered salicylic acid for a trial of three rheumatic patients. See Table III for medication scheme; simultaneously administered drugs: patient 1 (female), prednisone; patient 2 (male), nihil; patient 3 (female), prednisone, glibenclamide, pentazocine.

a series A, B and C blood samples were taken at time intervals of 1/4, 1/2, 1, 2, 4 and 8 h after the administration of the morning dose at 8.00 a.m. During this 8-h period the urine was collected to determine the total amount of the drugs, their metabolites and their glucuronides (see Fig. 1).

The pharmacokinetic curves of indomethacin and salicylic acid are shown in Figs. 4 and 5. The results for indomethacin show some changes in the area under the curves and the half-time and height of the maximum serum concentration.

Series A can be compared to series B (e.g. in the absence and in the presence of concurrently administrated salicylic acid). For patients 1 and 2 an increase of the area under the pharmacokinetic curve and of the maximum serum concentration of indomethacin was found going from A to B, and for patient 3 a decrease was found. For patients 1 and 3 the half-time remained 2 and 1 h,

TABLE III

MEDICATION SCHEME TO STUDY THE EFFECT OF CONCURRENT ADMINISTRATION OF INDOMETHACIN AND SALICYLIC ACID

Series	Day	Drugs*
A	1	50 mg indomethacin
	2	
	3	
	4**	
B	1	50 mg indomethacin +
	2	1 g sodium salicylate
	3	
	4**	
C	1	50 mg indomethacin
	2	
	3	
	4**	

*Simultaneously administered orally, three times daily at 8.00 a.m., 4.00 p.m. and 12.00 p.m. 50 mg indomethacin in capsule and 10 ml buffered medicine containing 1 g sodium salicylate.

**After the morning dose of 8.00 a.m. blood samples were taken at 8.15, 8.30, 9.00, 10.00, 12.00 a.m. and 4.00 p.m.

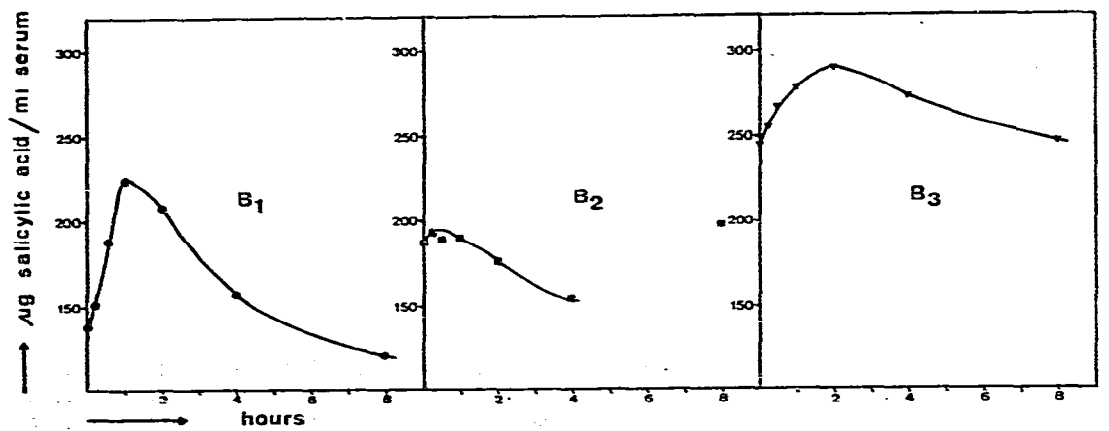

Fig. 5. Pharmacokinetic curves of salicylic acid for series B of the medication scheme.

respectively, while for patient 2 a shift from 0.5 to 1 h was found.

Comparing series C (e.g. in the absence of salicylic acid) to series A and B, the same curves were expected as for series A. However, for patients 1 and 2 the area and maximum increased again compared to series B, while for patient 2 a further shift of the half-time was found. For patient 3 the medication had to be stopped because of side effects.

The differences between the results for each individual patient are also illustrated by the pharmacokinetic curves of salicylic acid (see Fig. 5). It should be noticed that no salicylic acid was found in the serum samples of series C_1 and C_2.

The metabolites of indomethacin such as DMI, DBI, DMBI and 4-chloro-

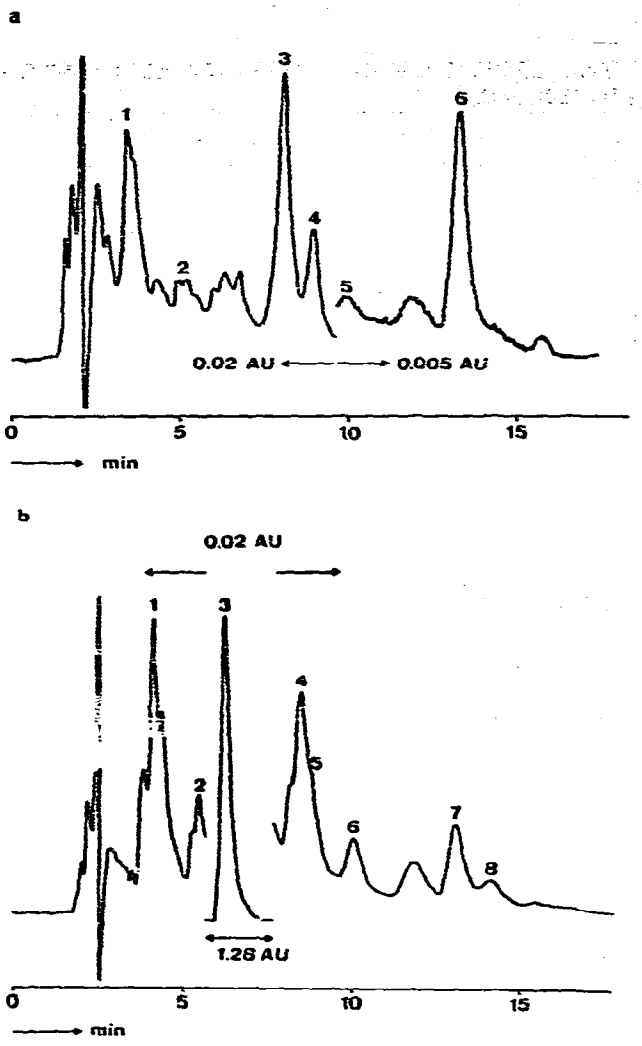

Fig. 6. Chromatograms of extracted serum, taken after oral administration of indomethacin in the presence and in the absence of salicylic acid. Conditions as in Fig. 3. (a) Series C_1 at 8.00 a.m.; peaks: 1, DMBI; 2, DBI; 3, serum background; 4, DMI; 5, 4-chlorobenzoic acid; 6, I. (b) Series B_3 at 10.00 a.m.; peaks: 1, DMBI; 2, DBI; 3, SA; 4, serum background; 5, DMI; 6, 4-chlorobenzoic acid; 7, I; 8, glibenclamide.

benzoic acid can be determined simultaneously with indomethacin and salicylic acid, as shown in Fig. 6a and b. However, the analytical results for the quantitative determination of metabolites in serum were for indistinct reasons less reproducible than for indomethacin and salicylic acid.

The collected urine samples were tested for the presence of the drugs, their metabolites and their glucuronides. As shown in Fig. 7a and b, a considerable concentration of metabolites was found in urine. From the peak heights before and after treatment of the urine with β-glucuronidase the concentrations of the free compounds and their glucuronides were calculated. As shown in Table IV, the relative abundance depends on the kind of compound.

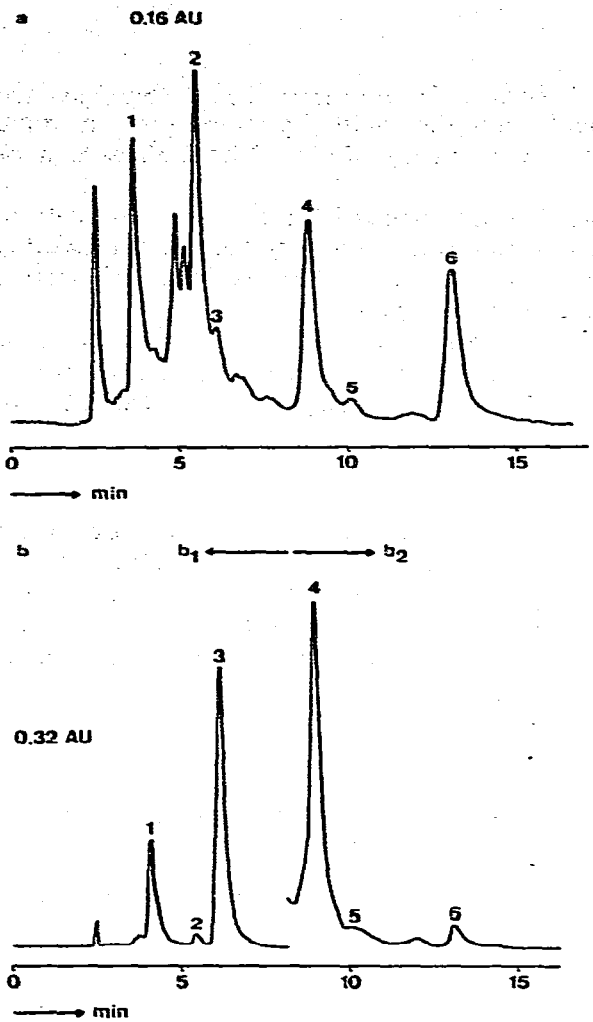

Fig. 7. Chromatograms of extracted urine, collected during the 8-h period of a pharmacokinetic curve in the presence and in the absence of salicylic acid. Conditions as in Fig. 3, (a) Series A_3; peaks: 1, DMBI; 2, DBI; 3, pentazocine; 4, DMI; 5, 4-chlorobenzoic acid; 6, I. (b) Series B_3; for part b_1 of the chromatogram the urine extract of part b_2 was 50 times diluted with the eluent; peaks: 1, SU; 2, DBI; 3, SA; 4, DMI; 5, 4-chlorobenzoic acid; 6, I.

TABLE IV

THE FREE COMPOUNDS AND THEIR GLUCURONIDES IN URINE

Compound	Glucuronide	Ratio
$\text{Indomethacin}_{free}$	$\text{Indomethacin}_{gluc}$	1:3
$\text{Salicylic acid}_{free}$	$\text{Salicylic acid}_{gluc}$	1:0.7
DMI_{free}	DMI_{gluc}	1:10
DBI_{free}	DBI_{gluc}	1:2

CONCLUSIONS

Only a small volume of blood serum is required to determine low concentrations of indomethacin and salicylic acid by means of a rapid extraction procedure and subsequent analysis by HPLC. Metabolites can be determined simultaneously.

The method is simple and reliable. The time needed for one analysis is about one hour. The stability of the chromatographic phase system is remarkably high: no change was noticed after injection of 2000 extracts of serum. Therefore, the method is suitable for routine analysis.

The results of the present pharmacokinetic study do not solve the clinical issue of a possible interaction of indomethacin and salicylic acid. Future research will be devoted to this subject for a larger group of rheumatic patients.

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