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High-performance liquid chromatographic determination of loxoprofen and its diastereomeric alcohol metabolites in biological fluids by fluorescence labelling with 4-bromomethyl-6,7-methylenedioxycoumarin

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ABSTRACT

A simple and sensitive high-performance liquid chromatographic procedure to determine loxoprofen and its diastereomeric alcohol metabolites in biological specimens is described. The analysis involves liquid–liquid extraction with benzene, pre-column derivatization with a highly fluorogenic reagent, 4-bromomethyl-6,7-methylenedioxycoumarin (BrMDC) and subsequent separation on a reversed-phase column. Loxoprofen, its pharmacologically active metabolite, *trans*-alcohol, and less active *cis*-alcohol were completely separated within 20 min with a mobile phase of 55% of aqueous acetonitrile containing acetic acid. Any endogenous substances do not interfere in the analysis of either plasma or urine samples. The quantitation limit was 0.01 μg/ml for human plasma and 0.05 μg/ml for urine. The method was applied to a pharmacokinetic study in healthy human subjects who had received 60 mg of loxoprofen sodium.

INTRODUCTION

Loxoprofen sodium [sodium (±)-2-[4-(2-oxocyclopentylmethyl)phenyl]propionate dihydrate; Loxonin®] is a 2-phenylpropionate non-steroidal antiinflammatory agent (NSAID) with marked analgesic and antipyretic activities and relatively weak gastrointestinal ulcerogenicity [1,2]. It has been shown that loxoprofen itself has only weak inhibitory activity against *in vitro* prostaglandin synthesis and that its pharmacological effect is derived from the rapid conversion of the cyclopentanone moiety into a corresponding active cyclopentanol metabolite, *trans*-alcohol, in the systemic circulation [3,4]. This reductive metabolism and the subsequent glucuronide conjugation are considered to be the major metabolic pathway of loxoprofen in humans [5].

Loxoprofen has two chiral centres in its molecule and the pharmaceutical products were marketed as a racemate equally containing four individual enantiomers. Thus, the alcohol metabolite is possibly made up of eight optically active enantiomers. However, extensive metabolic studies on loxoprofen have

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suggested that the stereoselective reduction at the cyclopentanone moiety toward 2′*S*-isomer of cyclopentanol and the metabolic inversion at the C-2 asymmetric carbon of the phenylpropionic acid from 2*R*- to 2*S*-epimers, like most other analogues, occur concomitantly during the primary process of metabolism [3,6–9]. Consequently, the overall metabolic reaction has a bias toward the favorable formation of (2*S*)-2-[4-(*trans*-(1′*R*,2′*S*)-2′-hydroxycyclopentylmethyl)-phenyl]propionic acid, [2*S*-*trans*-(1′*R*,2′*S*)]-alcohol, which has the most potent inhibitory activity of the eight possible enantiomers of the alcohol metabolite. Therefore, it seems to be necessary to quantify the alcohol metabolite as its individual enantiomers in order to obtain precise information about the pharmacokinetic and/or pharmacodynamic behaviour of loxoprofen. However, such enantioselective metabolism in rats and humans was proved to be very rapid and highly preferential [6,8,9]. In comparative pharmacokinetic studies or bioequivalence tests, therefore, it might still be of practical value to evaluate the parent acid and the diastereomeric *trans*-alcohol as the racemic species in biological specimens by some sort of conventional non-enantioselective technique.

In early clinical studies, loxoprofen and the corresponding diastereomeric alcohol metabolites in plasma and urine were determined after oral administration to healthy humans by gas chromatography combined with electron-impact ionization mass spectrometry (GC–MS) [5]. However, the overall analytical procedure was so tedious that it was inadequate for routine analysis in non-specialized laboratories. Furthermore, only partial resolution of the two diastereomeric alcohols, *trans* and *cis*, could be achieved under those conditions. An enantiospecific assay by high-performance liquid chomatography (HPLC) with fluorescence detection for the quantitative analysis of loxoprofen and its reductive metabolites in biological samples is also available [6]: a chiral reagent, (1*S*)-1-(4-dimethylamino-naphthalene-1-yl)ethylamine is coupled with the 2-phenylpropionic acid of the drugs to form the corresponding diastereoamides. It is usually necessary to separate each diastereomer in a normal-phase mode.

This paper describes the use of an achiral pre-column derivatization reagent for carboxylic acids, 4-bromomethyl-6,7-methylenedioxycoumarin (BrMDC) [10], for the selective and sensitive assay of loxoprofen and its corresponding diastereomeric alcohol metabolites in biological fluids using a convenient reversed-phase HPLC procedure.

EXPERIMENTAL

Materials

All solvents and chemicals were of analytical-reagent grade unless noted otherwise. Loxoprofen and its diastereomeric alcohol metabolites, (±)-2-[4-(*trans*-2-hydroxycyclopentylmethyl)phenyl]propionic acid (*trans*-alcohol) and (±)-2-[4-*cis*-2-hydroxycyclopentylmethyl)phenyl]propionic acid (*cis*-alcohol), were obtained from Chemical Research Labs. of Sankyo (Tokyo, Japan) [2,11,12]. These

reference compouds were dissolved together in methanol to give a final concentration of 1 mg/ml, and stored at 4°C. Loxonin was the commercially available tablet supplied by Sankyo, which contained 60 mg of loxoprofen sodium as the dihydrate. BrMDC was prepared according to our previous report [10]. 1-Naphthoic acid, used as the internal standard, was purchased from Wako (Osaka, Japan).

Extraction and derivatization procedure

Human plasma or nine-fold diluted urine with water (each 0.5 ml) was acidified with 0.2 ml of 2 *M* hydrochloric acid. 1-Naphtoic acid (2 μg/ml, 0.5 ml) was added as internal standard. The aqueous mixture was then extracted twice with 6 ml of benzene. After centrifugation at 900 *g* for 10 min, the upper organic layer was transferred to another conical tube and evaporated to dryness *in vacuo*. To the residue were added 0.5 ml each of an acetonitrile solution of BrMDC (250 μg/ml) and 18-crown-6 (1.5 mg/ml). Each solution was made on the day of the experiments, and the crown ether was saturated with an excess of finely powdered potassium carbonate and sonicated briefly prior to use. Subsequently, the mixture was heated at 40°C for 30 min to esterify the propionyl moiety of loxoprofen and its metabolites, as shown in Fig. 1. A 0.1-ml volume of acetic acid–acetonitrile (1:9, v/v) was added in order to terminate the reaction and stabilize the ester derivatives formed.

For the determination of the amounts excreted as ester-glucuronide, a 0.5-ml aliquot of diluted urine was hydrolysed with an equal volume of 0.5 *M* sodium hydroxide prior to solvent extraction. Treated urine was neutralized with 0.5 *M* hydrochloric acid after standing for 30 min at ambient temperature.

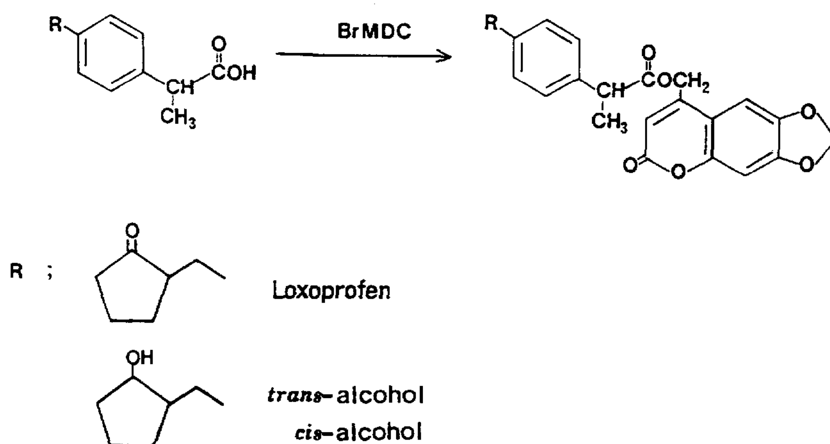


Fig. 1. Derivatization of loxoprofen and its diastereomeric alcohol metabolites with BrMDC.

Calibration curves

Drug-free plasma or diluted urine containing authentic loxoprofen and its diastereomeric alcohols (as free acid) to give final concentrations of 0.31–5 μg/ml were analysed as described above. The peak-area ratio of each compound to the internal standard was plotted against concentration. Then a linear least-squares analysis was performed with a model equation of $y = ax + b$. The concentrations in unknown samples or drug-spiked samples for validation studies were obtained from the slopes and the intercepts of these standard curves.

Chromatography

HPLC was carried out with a Model 655 solvent-delivery system (Hitachi, Tokyo, Japan) equipped with a Model F-1000 fluorescence spectrophotometer (Hitachi), which was operated at an excitation wavelength of 355 nm and an emission wavelength of 435 nm. A 5-μl aliquot of the reaction mixture was introduced on a stainless-steel Zorbax ODS column (250 mm × 4.6 mm I.D., DuPont, Wilmington, DE, U.S.A.) through a Model WISP 710B automatic sample processor (WISP 710B, Waters, Milford, MA, U.S.A.). The mobile phase, acetonitrile–water–acetic acid (55:45:1, v/v), was routinely filtered and degassed by sonication immediately before use. Isocratic flow-rates of 1.2 ml/min for plasma samples and 1.5 ml/min for urine samples were used. Peak areas were determined with a Model C-R3A digital integration recorder (Shimadzu, Kyoto, Japan).

Clinical study

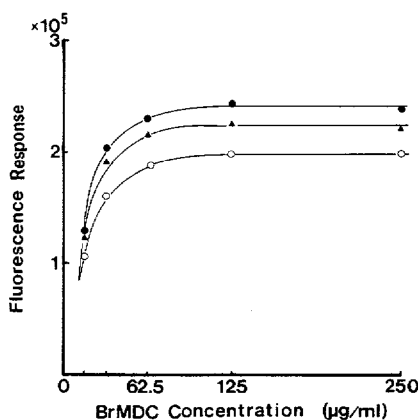
Sixteen healthy male volunteers, age 46.8 ± 3.7 years and weight 63.9 ± 3.7 kg, fasted overnight prior to the experiment. Single oral doses of 60 mg Loxonin in tablet form were given at 9:00 a.m. Blood samples (each 5 ml) were withdrawn by venipuncture using heparinized collecting tubes (Venoject®, Terumo, Tokyo, Japan) at 0, 0.25, 0.5, 1, 1.5, 2, 4 and 6 h after dosing. Each plasma portion was obtained by centrifugation and stored at −20°C until analysis. Another six healthy volunteers, age 46.3 ± 2.3 years and weight 63.0 ± 2.6 kg, were given oral doses of the same formulation of loxoprofen sodium for the purpose of the urinary excretion study. Urine samples were collected at designated intervals for up to 8 h. Each subject was given a glass of water (200 ml) just after sample collections in order to enhance urine flow.

Data analysis

The following pharmacokinetic parameters were calculated by a model-independent moment analysis from observed plasma levels after oral dose of loxoprofen: the area under the concentration–time curve (AUC), elimination half-life ($t_{1/2}$), mean residence time (MRT), peak concentration (C_{max}) and time to reach peak concentration (t_{max}).

RESULTS AND DISCUSSION

In our previous paper, we described how loxoprofen and its diastereomeric



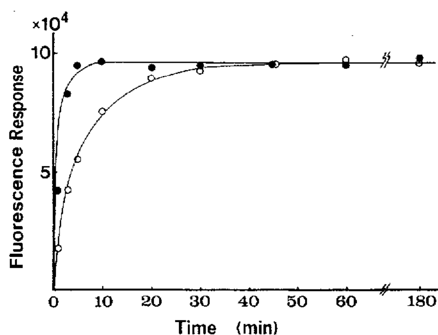


Fig. 2. Effect of the concentrations of BrMDC on the formation of fluorogenic esters with loxoprofen (●), *trans*-alcohol (○) and *cis*-alcohol (▲). Drug-free plasma samples were spiked with loxoprofen and two diastereomeric metabolites (each 5 µg/ml), extracted and derivatized with BrMDC. The fluorescence yield is expressed as the peak-area count calculated by an integrator.

Fig. 3. Time-course for derivatization of loxoprofen with BrMDC: 2 µg/ml authentic loxoprofen was treated with 250 µg/ml BrMDC in the presence of alkaline crown ether at 40°C (●) or ambient temperature (○).

alcohol metabolites in rat liver cytosol preparations could be completely separated as their methyl esters by normal-phase HPLC during pretreatment for the resolution of the 1′,2-enantiomers by capillary GC–MS [8,9]. However, their weak UV absorption and some interfering peaks might spoil the sensitivity for *in vivo* preparations, *i.e.* plasma or exudate obtained from an inflamed tissue. Hence, we used an achiral fluorescence labelling reagent for carboxylic acids, BrMDC [10], which results in the improvement of selectivity and sensitivity. Solid-phase extraction using an ODS mini-column (Sep-Pak C_{18}) gave a high recovery of *trans*-alcohol and *cis*-alcohol in rat liver preparations [8]; however, we preferred to use repetitive solvent extraction with benzene from acidified samples. Each compound (5 µg/ml) was effectively recovered from human plasma with an extraction ratio of 95% for loxoprofen, 80% for *trans*-alcohol and 89% for *cis*-alcohol, respectively.

The plasma extract containing 5 µg/ml loxoprofen and its two diastereomeric alcohol metabolites was derivatized with different concentrations of BrMDC, in the presence of excess K_2CO_3 and a phase-transfer catalyst in order to ascertain the optimal amount of reagent required. Although authentic *n*-caproic acid was esterified sufficiently with an equivalent amount of reagent in a molar stoichiometric fashion [10], at least a six-fold excess (125 µg/ml) of BrMDC was needed for the reaction to go to completion (Fig. 2). This might be attributable to endogenous components, such as free fatty acids or their glycerides, being simultaneously extracted with the acidified organic solvent and competing for reagent. Therefore, we used an excess amount (250 µg/ml) for subsequent studies.

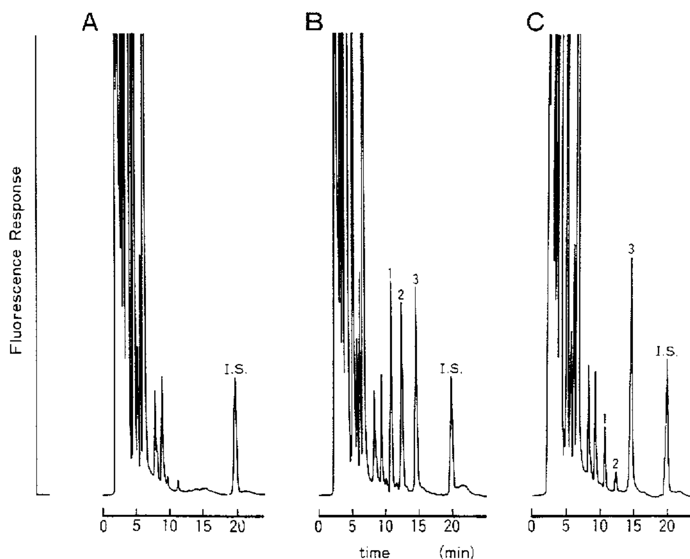


Fig. 4. Chromatograms of extracts from human plasma. (A) A drug-free sample containing only the internal standard; (B) a sample containing 2.5 μg/ml each of authentic standards; (C) a sample obtained 1 h after an oral dose of loxoprofen sodium as a 60-mg tablet of Loxonin. Peaks: 1 = *trans*-alcohol; 2 = *cis*-alcohol; 3 = loxoprofen; I.S. = internal standard.

The reaction time required to obtain the maximum yield of fluorescence was as short as 40 min even at ambient temperature (Fig. 3). However, we used 40°C for 30 min, because the internal standard 1-naphthoic acid did not react completely

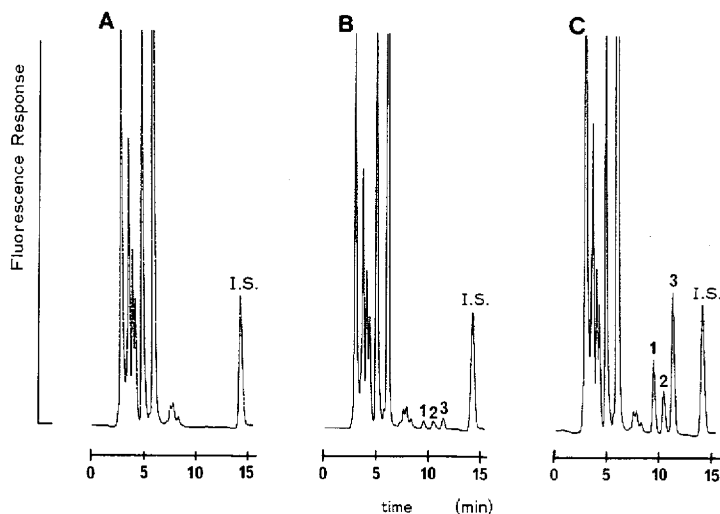


Fig. 5. Chromatograms of extracts from human urine. (A) A drug-free sample containing only the internal standard; (B) and (C) a sample before (B) or after (C) alkaline hydrolysis obtained 1 h after an oral dose of loxoprofen sodium as a 60-mg tablet of Loxonin. Peak numbers as in Fig. 4.

TABLE I

ACCURACY AND REPRODUCIBILITY FOR THE ANALYSIS OF LOXOPROFEN AND ITS ALCOHOL METABOLITES IN HUMAN PLASMA AND URINE

Sample	*n*	Spiked (μg/ml)	Found (μg/ml)			Coefficient of variation (%)		
			Loxoprofen	*trans*-Alcohol	*cis*-Alcohol	Loxoprofen	*trans*-Alcohol	*cis*-Alcohol
Within-assay								
Plasma	10	5.0	4.90	5.04	4.91	5.7	6.0	4.7
	10	1.0	1.05	1.13	1.06	4.8	8.0	6.6
	10	0.5	0.51	0.47	0.48	3.9	6.4	4.2
Urine	5	20.0	19.7	20.4	20.6	2.6	2.1	2.8
Between-assay								
Plasma	4	5.0	4.78	4.74	4.92	9.3	10.5	12.2
Urine	4	20.0	21.2	19.1	19.7	5.4	6.5	6.3

at lower temperature. A small amount of acetic acid was also needed to consume the remaining reagent, which otherwise might result in the undesirable decomposition of adducts formed and large inter- or intra-assay variations.

A wide variety of HPLC stationary phases (μBondapak C_{18}, Partisil 5-ODS, Cosmosil $5C_{18}$, Zorbax ODS and LiChrosorb RP-18) were tested for their selectivity and resolution for the *trans*- and *cis*-alcohols. The best separation was obtained with Zorbax ODS; an excellent resolution factor resulted with a mobile phase of 55% acetonitrile containing 1% acetic acid. Typical chromatograms of extracts from a human plasma and urine are shown in Figs. 4 and 5, respectively. The flow-rate was varied depending on the sample, because plasma extracts often contained rather larger interference peaks than those of diluted urine.

The calibration curves for each derivatized compound in plasma samples were almost linear in the concentration range 0.31–5 μg/ml. Typical curves for each compound were described by the following equotations: $y=0.573x-0.005$ for loxoprofen; $y=0.471x-0.012$ for *trans*-alcohol; $y=0.489x-0.004$ for *cis*-alcohol. The correlation coefficients were larger than 0.999 for all compounds. A similary good linearity was also found for urine samples.

The accuracy, precision and reproducibility of the method were examined with spiked plasma and urine samples (Table I). The within-assay coefficients of variation (C.V.) were less than 8.0% for plasma and 2.8% for urine samples. In spite of slightly larger variations in the concentrations found when assayed on four different periods over two weeks, the method remains valid. The minimum quantifiable concentration of each derivatized compound was 0.01 μg/ml in plasma and 0.05 μg/ml in urine, at a signal-to-noise ratio of 3. These are similar to the values with GC–MS [5].

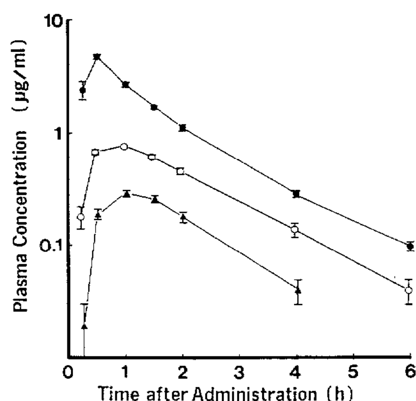


Fig. 6. Mean plasma concentration–time curves of loxoprofen and its diastereomeric alcohols after an oral dose of loxoprofen sodium as a 60-mg tablet of Loxonin to healthy humans. Each value is expressed as the mean ± S.E. of sixteen subjects. Symbols as in Fig. 2.

The pharmacokinetics of loxoprofen in healthy humans were investigated using the present method. The average plasma concentrations and pharmacokinetic parameters of loxoprofen and the corresponding alcohol metabolites in sixteen subjects who received a single oral dose of loxoprofen sodium are shown in Fig. 6 and Table II, respectively. Loxoprofen was rapidly absorbed, and the highest plasma level of 4.92 μg/ml was achieved 31 min after the dose. This declined bi-exponentially with an elimination half-life of 1.15 h. Plasma concentrations of *trans*- and *cis*-alcohol were lower than those of loxoprofen at all times, where the area under the curve (AUC) ratio of each to unchanged loxoprofen was counted as one third or one tenth, respectively. Time-courses of urinary excretion of loxoprofen and its metabolites as total species (free + conjugate) are shown in Fig. 7. The cumulative excretion of loxoprofen and metabolites up to 8 h post-dose increased *ca.* ten-fold following treatment with alkali (Fig. 5). The ratio of both

TABLE II

MODEL-INDEPENDENT PHARMACOKINETIC PARAMETERS OF LOXOPROFEN AND THE CORRESPONDING REDUCTIVE METABOLITES AFTER AN ORAL DOSE OF LOXOPROFEN SODIUM TO HEALTHY HUMANS

Parameter	Loxoprofen	*trans*-alcohol	*cis*-alcohol
AUC (μg · h/ml)	6.70 ± 0.26[a]	2.02 ± 0.05	0.68 ± 0.06
$t_{1/2}$ (h)	1.15 ± 0.07	1.34 ± 0.07	1.13 ± 0.10
MRT (h)	1.57 ± 0.05	2.29 ± 0.08	2.09 ± 0.12
C_{max} (μg/ml)	4.92 ± 0.22	0.81 ± 0.02	0.32 ± 0.02
t_{max} (h)	0.52 ± 0.04	0.84 ± 0.06	1.03 ± 0.06

[a] Mean ± S.E. of sixteen subjects.

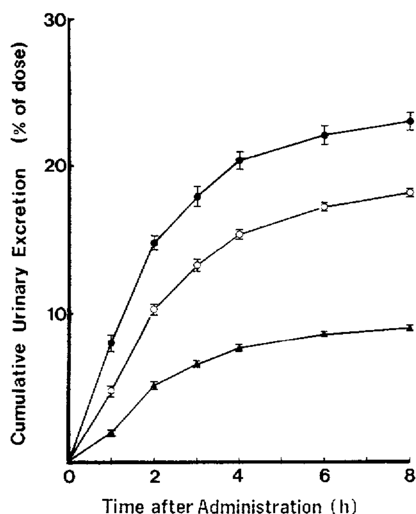


Fig. 7. Cumulative urinary excretion (free + conjugate) of loxoprofen and its diastereomeric alcohols after an oral dose of loxoprofen sodium as a 60-mg tablet of Loxonin to healthy humans. Each value is calculated as the percentage of the dose equivalent to the parent acid, and expressed as the mean ± S.E. of six subjects. Symbols as in Fig. 2.

alcohol metabolites to loxoprofen were higher in urine than in plasma: 80% for *trans*-alcohol and 40% for *cis*-alcohol. This may be because the quantitative renal handling of loxoprofen was somewhat different from that of alcohol metabolites, and/or because reductive metabolism to the alcohols occurs in liver as well as other tissues, including the kidney [13]. The above results correspond well with the pharmacokinetic behaviour observed during the Phase I clinical trial of loxoprofen [5].

In conclusion, this fluorescence HPLC technique can be used to quantify both loxoprofen and its pharmacologically active metabolites simultaneously in clinical plasma and urine samples. To date the assay has been applied to *ca.* 5000 samples, including animal experiments. The satisfactory reliability and sensitivity of the method may provide some important knowledge of the clinical pharmacology of loxoprofen in patients with chronic disorders. One of typical applications in progress is to investigate the comparative tissue penetration of NSAIDs [14]. Slight modifications of the method enable the determination of trace concentrations of loxoprofen and its metabolites in synovial fluids obtained from drug-treated patients with rheumatoid arthritis.

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