High Performance Liquid Chromatographic Determination of Diclofenac Sodium in Plasma Using Column-switching Technique for Sample Clean-up

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For routine analysis of diclofenac sodium in plasma, a new high performance liquid chromatographic method, which is combined with column-switching technique is developed. The precolumn packed with Corasil RP C-18 was connected to analytical column by switching system in order to enrich the sample drugs in plasma without extraction.
This method showed excellent sensitivity, precision and reproducibility. The limit of detection, using a 100 µL injection of plasma, was 0.1 µg/mL and the mean coefficient of variation for intra- and inter-assay was better than 4.6%. Total analysis time was 20 min between injections.
The present method offers distinct practical advantages over conventional liquid-liquid extraction methods of sample preparation with respect to time, effort, recovery, and sample volume required. The method has been applied to the samples from rats receiving oral administration of diclofenac sodium.

HPLC-Bestimmung von Diclofenac-Na im Plasma durch Column-switching Technik zur Probenaufbereitung

Beschrieben wird eine neue Hochdruck-Flüssigkeits-Chromatographie-Methode mit Hilfe der Column-Switching Technik für die Bestimmung von Diclofenac-Na in Plasma. - Das Verfahren umfaßt eine automatische Festphasen-Extraktion auf Corasil RP C-18 Extraktionssäulen. - Die Methode ist durch hohe Empfindlichkeit, Präzision und Reproduzierbarkeit charakterisiert. Die untere Nachweisgrenze lag bei 0.1 µg/mL bei der Injektion von 100 µL Plasma, der durchschnittliche Variationskoeffizient für Intra-, Inter-Assay war besser als 4.6%. Die gesamt Analyse dauerte etwa 20 min.
Die hier beschriebene Methode zeigte praktische Vorteile in Bezug auf Schnelligkeit, Wiederfindung, Probenvorbereitung und Probenvolumen gegenüber der konventionellen Flüssig-Flüssig-Extraktion. - Eingesetzt wurde die Methode zur Bestimmung von Proben mit Diclofenac-Na behandelter Ratten.

Diclofenac sodium (DCF-Na) is a non-steroidal antiinflammatory drug used in the treatment of rheumatic diseases[1]. Several methods have been published for the determination of DCF-Na in plasma or serum. Among them are thin layer chromatography (TLC)[2], gas chromatography (GC)[3-6] and high performance liquid chromatography (HPLC)[7-12].

The GC methods are very sensitive; however, they require large sample volumes, derivatization steps and extensive sample clean-up. The HPLC methods employ liquid-liquid extractions prior to chromatography. These extractions involve an initial acidification step, extraction with org. solvents and evaporation to dryness followed by reconstitution in methanol or mobile phase. All the processes are time-consuming and are prone to introduction of errors.

Recently, precolumn techniques for the enrichment of drugs in biological fluids without pretreatment have been developed in order to increase sample throughput and accuracy. The precolumn has been used off-line[13] or on-line[14-19] in a chromatographic column switching system.

The objective of the present study was to develop a HPLC method for the determination of DCF-Na in plasma that is more sensitive, selective, reproducible and convenient than conventional methods in terms of sample handling and speed of analysis. The present paper describes precolumn-switching technique which allows on-line sample loading and rapid elution of the analytes from a precolumn and direct analysis on the analytical column.

Materials and Methods

Reagents and standards

Acetonitrile (p.chr.), methanol (p.chr.), NaH_2PO_4 (p.a.), Na_2HPO_4 (p.a.) and H_3PO_4 (p.a.) were obtained from E. Merck (Darmstadt, F.R.G.). Water was distilled and then deionized with Nanopure II (Barnstead).

DCF-Na and N-phenylanthranilic acid (NPA) were obtained from Sigma Chemical Co. (St.Louis, MO, U.S.A.) and Tokyo Kassei Co. (Tokyo, Japan), respectively.

Stock solutions were prepared in methanol (1 mg/mL) and stored at 4°C. This stock solution was diluted with 0.05 M phosphate buffer (pH 2) as necessary and used to prepare the appropriate concentrations (2.0 - 200 µg/mL for DCF). NPA (internal standard) was used as 15 µg/mL standard solution in phosphate buffer (0.05 M, pH 2.0). Spiked plasma standards ranging 0.1 - 10 µg/mL of DCF in plasma were then prepared in each assay by spiking 1 mL of plasma with 100 µL of DCF working standards. 100 µL of internal standard solution was added to spiked plasma samples.

Instrumentation

The HPLC system consisted of a Spectra Physics Model SP 8800 pump (SantaClara, CA, U.S.A.), a Waters 501 pump (Waters Assoc., Milford, MA, U.S.A.), a Rheodyne 7125 injector (Cotati, CA, U.S.A.), a Rheodyne 7000 switching valve and a Spectra Physics SP8450 UV/VIS detector. Chromatogram recording and peak integrations: Spectra Physics SP4290 integrator.

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Precolumn was a 2 x 0.4 cm i.d. stainless steel column (Waters Assoc.) dry-packed with Corasil RP C-18 (37-53 μm, Waters Assoc.). The analytical column was a 25 x 0.46 cm i.d. stainless steel column prepacked with 10 μm Lichrosorb RP-18 (Spectra Physics).

Chromatographic conditions

The instrument arrangement is shown in Fig. 1. The washing solvent was phosphate buffer (0.05 M, pH 2.0) and the flowrate was 0.5 mL/min for 4 min after injection of sample, and then, 1.0 mL/min. The mobile phase was 30% acetonitrile in phosphate buffer (0.05 M, pH 7), flowrate 1.0 mL/min. Temp.: ambient, wavelength of the UV detector: 280 nm.

Column-switching procedure for sample handling

The plasma sample was injected into the precolumn which was washed at a flowrate of 0.5 mL/min for 4 min and then of 1.0 mL/min for 4 min. The drugs were adsorbed on the precolumn, while other components in plasma were drained away. During washing of the precolumn, the mobile phase from pump B was flowed into the analytical column, and the switching valve was turned to the mobile phase at the 8. min after injection of the plasma sample. The drugs enriched on the precolumn were eluted into the analytical column by back-flush mode and separated efficiently. The valve was returned to its initial position after 4 min when DCF and NPA were eluted completely into the analytical column.

System characterization

Breakthrough studies of DCF on Corasil RP C-18 were performed by connecting the outline of the precolumn directly to the detector as shown in Fig. 1. Aliquots of either 10, 20 or 40 μg/mL DCF standard solution were injected, and 100 mL of phosphate buffer (0.05 M, pH 2.0) was then passed through the precolumn at a flowrate of 3 mL/min prior to elution with the mobile phase at a flowrate of 1.0 mL/min. The effluents were continuously monitored for DCF breakthrough. The plate numbers of the eluted peaks before and after washing the precolumn were determined.

The effect of the washing solvent flowrate on the recovery of DCF from spiked plasma samples was evaluated by comparing the relative peak areas of 10 μg/mL of DCF in a spiked plasma and an aqueous standard. Following each injection, 6 mL of washing solvent was passed through the precolumn at flowrates of either 0.5, 1.0, 1.5 or 2.0 mL/min before the drug was eluted by the mobile phase.

In order to determine whether the precolumn/switching valve was influencing the total band broadening of the LC system, 10 μL loop injections of DCF standard solution (80 μg/mL) and 100 μL trace enrichment of DCF on the precolumn after 10-fold dilution of DCF solution with phosphate buffer (0.05 M, pH 2.0) were compared. Elution after trace enrichment was carried out in both the forward-flush and the back-flush mode.

Results and Discussion

Choice of precolumn packing and washing solvent

It is necessary that precolumn packing and washing solvent have to be chosen in such a way that DCF is completely adsorbed while the interfering components in plasma are not adsorbed on the precolumn. Corasil RP C-18 is suitable as a precolumn packing because of its relatively strong adsorption properties and availability.

DCF is an acidic drug (pKa = 4.0), and, therefore, the capacity factor k' of DCF changes with pH (Fig. 2). The nonionic form of DCF is more predominated at lower pH. At pH 2.0, DCF is present as nonionic form and the charges of amino acid groups of albumin are changed. Consequently, the majority of plasma components is not selectively adsorbed on the precolumn in phosphate buffer (0.05 M, pH 2.0) and DCF exhibits strong retention on Corasil RP C-18. Therefore, phosphate buffer (0.05 M, pH 2.0) was used as washing solvent in this system.

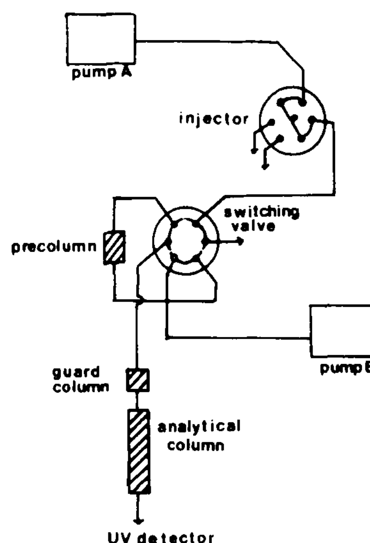

Fig. 1. Schematic diagram of a column-switching system.

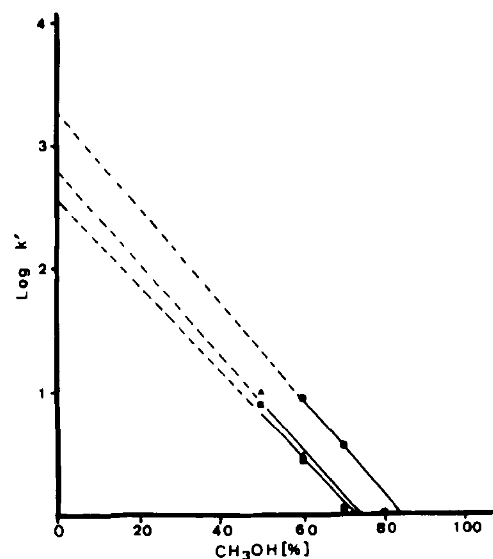

Fig. 2. Capacity factor (log k') of diclofenac as a function of mobile phase composition using methanol-water as the mobile phase. stationary phase: Corasil RP C-18 (37-53 μm), column: 10 x 0.4 cm i.d., flowrate: 1.0 mL/min, detection: UV 280 nm. ● pH 2.0 ▲ pH 4.5 ■ pH 7.0

Choice of analytical conditions

The coice of an octadecyl-silica packing for the analytical column was based on the high efficiency, ready availability, and popularity of such bonded phase packings.

Optimization of the separation leads to the use of the following mixture as the mobile phase : 30% acetonitrile in phosphate buffer (0.05 M, pH 7.0).

As DCF is present as an ionized form at pH 7.0, it is very soluble in the aqueous-organic mobile phase. Therefore, DCF can be eluted quantitatively from the precolumn.

Breakthrough studies

No breakthrough was observed after 100 mL of phosphate buffer (0.05 M, pH 2.0) was passed through Corasil RP C-18 column loaded by 4 µg of DCF (Fig. 3). This indicates that DCF was retained on the precolumn when washing solvent that washes off water-soluble interfering components of plasma was flowed sufficiently. Breakthrough volume of 4 µg DCF seemed to be higher than 100 mL when 120 mg of Corasil RP C-18 was used.

After breakthrough plots, the mobile phase was passed through the precolumn to elute the adsorbed DCF. The eluted peaks were gaussian shape with little tailing (Fig. 4). They exhibited nearly the same total plate numbers of DCF peaks of the direct mobile phase elution. This indicates that DCF was strongly retained in a narrow band on the top of the precolumn, and band broadening due to diffusion during the washing solvent passage was negligible. The mobile phase (2 mL) seems to be sufficient for the quantitative elution of DCF from the precolumn.

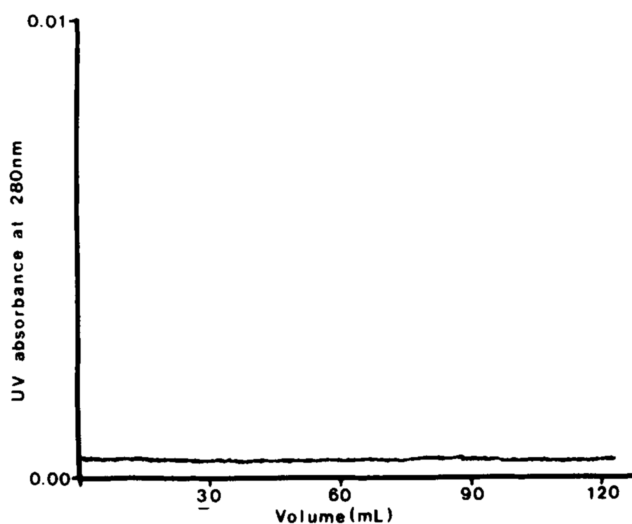

Fig. 3. Breakthrough plot of DCF on Corasil RP C-18.

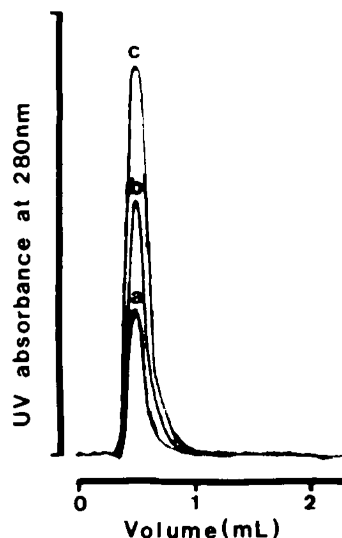

Fig. 4. Mobile phase elution curves of DCF. (a) 1 µg, (b) 2 µg and (c) 4 µg

Chromatography of plasma

The effectiveness of washing solvent in removing the plasma components from the precolumn is shown in Fig. 5 a. The chromatogram was obtained by injecting 100 µL of plasma sample, flushing 6 mL of washing solvent through the precolumn, and then, eluting with the mobile phase.

The large tailing peak resulted partly from the elution of plasma components which were not removed from the precolumn by washing solvent. However, injection of 100 µL of distilled water yielded a similar but smaller peak. Therefore, washing solvent flushed out by the mobile phase also contributed to this peak.

Influence of washing solvent flowrate

The influence of plasma on the uptake of DCF by the Corasil RP C-18 precolumn was evaluated by comparing the relative peak areas obtained from a spiked plasma and an aqueous solution containing 10 µg/mL DCF, respectively. As shown in Fig. 6, the recovery of DCF from plasma exhibits a high dependence on the flowrate of washing solvent.

This suggests that the adsorption of DCF on Corasil RP C-18 was kinetically slower in plasma than in water alone. A longer residence time was required during the adsorption step to ensure the quantitative uptake. It is known that more than 99.5% of DCF is very strongly bound to plasma proteins at therapeutic concentration levels[20].

The following two possible explanations of the slow uptake of DCF seen in Fig. 6 was consistent with the data: slow adsorption of DCF alone or DCF-protein complex on the Corasil RP C-18 surface and slow dissociation of DCF-protein complex. As shown in Fig. 6, the quantitative recovery of free and protein-bound DCF was achieved by passage through the precolumn under the correct conditions.

Influence of precolumn on extracolumn band broadening

Although it has been found that precolumn packed with particles of the same size as those of the analytical column produces very little extra-column band broadening[21], particles larger than 10 µm were used in the present study in order to avoid plugging when 10 µm particles were used. Therefore, overall column efficiency may be degraded below that of an analytical column/guard column. The loss in efficiency due to the precolumn can be minimized by using a backflush elution of drugs from the precolumn[15,18,19].

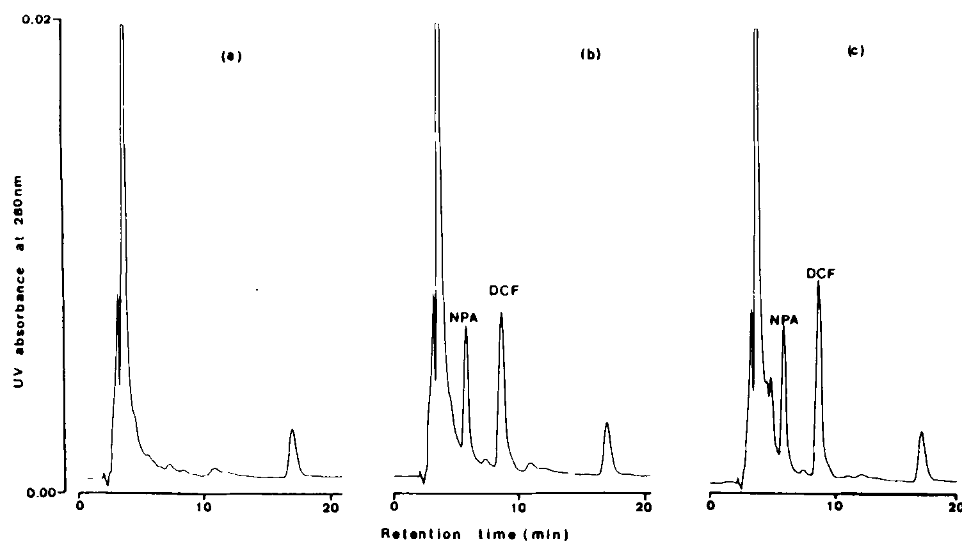

Fig. 5. Chromatograms of (a) a pooled rat drug-free plasma, (b) drug-free plasma spiked with internal standard and 4 µg/mL DCF-Na and (c) plasma of a rat 30 min after a single 10 mg/kg oral dose of DCF-Na.

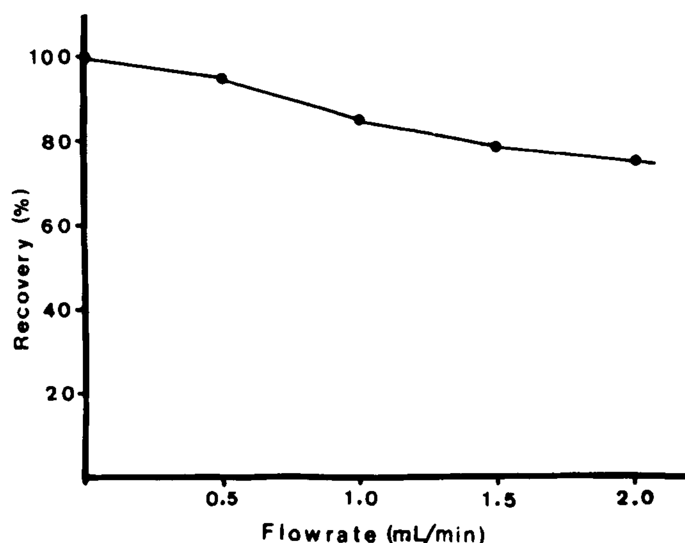

Fig. 6. Effect of washing solvent flowrate on the recovery of DCF from spiked plasma (10.0 µg/mL).

The results in Table 1 show that there is a decrease in plate number and a small increase in asymmetry factor caused by extracolumn band broadening. Remarkable difference between forward- and back-flush elution was also observed. This difference indicates that the retention on the precolumn during elution step is not negligible. Use of back-flush mode resulted in nearly the same efficiency and tailing as analytical column/guard column system alone.

Table 1. Effect of Direction of Precolumn Flow on Overall Efficiency (n=5)

	Plate number	Asymmetry factor
Direct injection	1870 ± 79	1.07 ± 0.07
Back-flush elution	1680 ± 135	1.08 ± 0.06
Forward-flush elution	1283 ± 64	1.18 ± 0.09

Recovery

In order to determine the recovery rate, two calibration curves based on the external standard method were plotted. One calibration curve was based on spiked plasma samples in concentration range 0.1 - 10.0 µg/mL and the other one was based on aqueous standards in the same range.

The overall recovery was calculated by the two methods: (i) comparison of the slopes of the regression lines for the two sets and (ii) direct comparison of the peak heights (Table 2).

As seen in Table 2, mean recovery rate from peak height was 97.3%, recovery rate from regression line was 92.6%, and overall mean recovery rate from two methods was 95.4%.

Linearity, limit of detection and precision

Evaluation of the assay was carried out using six point standards in concentration range 0.1 - 10 µg/mL DCF in plasma. The calibration plot of peak height ratios of drug/internal standard versus the concentration of DCF in plasma was linear with a correlation coefficient of 1.000.

Under the conditions described above, the limit of detection was 0.1 µg/mL DCF in plasma when 100 µL plasma was used. Detection limit was defined as the amount of compound showing a signal-to-noise ratio > 3:1.

Table 3 shows the within-batch(intra-assay) and between-batch(inter-assay) variation of the method. The precisions of the method (mean coefficient of variation, C.V.) were 3.5 and 4.6% for intra- and inter-assay, respectively.

Interference study

Other anti-inflammatory drugs were tested for possible interferences. The relative retention times of other anti-inflammatory drugs to DCF were 1.41 min for indomethacin,

Table 2. Recovery of DCF from Spiked Plasma

Concentration (µg/mL)	DCF peak heights (n=4) Set A:aqueous standard	Set B:standard extracted from plasma	Recovery (%)
0.1	307	317	103
0.5	1312	1285	97.9
1.0	2623	2557	97.5
2.0	5374	5218	97.1
5.0	12985	12372	95.3
10.0	27213	25144	92.7

Mean recovery: 97.3%
Regression line for set A: y = 2700.4 x - 84.7, r=1.000
Regression line for set B: y = 2501.4 x + 61.3, r=1.000
Mean recovery: (2501.4 / 2700.7) x 100 = 92.6%
Overall mean recovery determined by the two methods: 95.4%

Table 3. Precision, Accuracy and Reproducibility (n=4)

Concentration added (µg/mL)	Concentration found (µg/mL)	C.V.(%)	Bias(%)***
Intra-assay(Repeatability)*			
0.1	0.1 ± 0.01	9.1	0
0.5	0.5 ± 0.02	4.9	0
1.0	1.0 ± 0.01	1.5	0
2.0	1.9 ± 0.05	2.5	5.0
5.0	5.0 ± 0.05	0.9	0
10.0	9.3 ± 0.20	2.2	7.0
Mean		3.5	
Inter-assay(Reproducibility)**			
0.1	0.1 ± 0.01	7.0	0
0.5	0.5 ± 0.02	3.2	0
1.0	1.0 ± 0.06	6.0	0
2.0	1.9 ± 0.05	2.5	5.0
5.0	4.8 ± 0.25	5.3	4.0
10.0	9.2 ± 0.27	2.9	8.0
Mean		4.6	

* determination in quadruplicate
** single determination in four replicate run
*** bias : difference between added and found concentration

0.58 min for piroxicam, 2.13 min for mefenamic acid, 1.15 min for lonazolac, 0.94 min for phenylbutazone and 0.51 min for alclofenac. No drugs studied interfered with the determination of DCF.

Application study

The present method has been successfully applied to the samples from rats receiving an oral administration of DCF. A chromatogram from an actual plasma sample is shown in Fig. 5. This chromatogram resembles the chromatogram obtained from spiked plasma, and no interferences of endogenous plasma components were observed.

Comparison of the results obtained by the present method and those obtained by the conventional liquid-liquid extraction of *Godbillon* et al.[9] has been made for the determination of DCF in 18 rat plasma samples (Fig. 7). The correlation coefficient was 0.987 which can be considered as quite satisfactory.

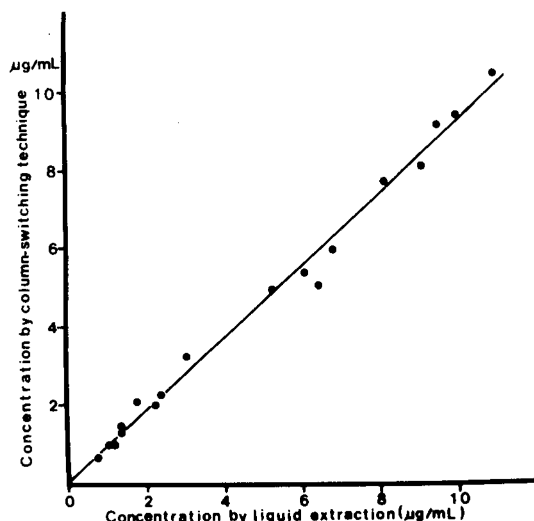

Fig. 7. Comparison of the present column-switching method and the conventional liquid-liquid extraction method for assaying DCF in plasma samples from rats. The line depicts x = y. When the results were analyzed by the least-squares method a slope of 0.919 and x intercept of -0.03, with r = 0.987, were obtained.

In conclusion, the present study demonstrated that column-switching technique using Corasil RP C-18 has a good advantage for purification and concentration of DCF-Na with a high recovery rate from plasma without laborious liquid-liquid extractions. Total analysis time was 20 min between injections. It is recommendable that the precolumn should be changed after every 100 injections. The present method might be further extended to other acidic non-steroidal anti-inflammatory drugs in plasma.

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