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Liquid chromatographic determination of diclofenac in human synovial fluid

Robert Roškar*, Vojko Kmetec

Faculty of Pharmacy, University of Ljubljana, Aškerčeva 7, 1000 Ljubljana SI-1000, Slovenia

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Abstract

A simple, rapid and sensitive HPLC method for the determination of diclofenac in synovial fluid is described. Special attention was paid to the procedure of sample preparation since gel formation may sometimes occur in synovial samples. With a one-step extraction procedure good and reproducible recovery of diclofenac was obtained. A subsequent HPLC assay was adjusted so as to achieve adequate sensitivity and precision needed for analysis of true samples. The results obtained by the described procedure proved the method to be suitable for monitoring concentrations of diclofenac in synovial fluid.

Keywords: Diclofenac; Synovial fluid

1. Introduction

Diclofenac sodium is a non-steroidal anti-inflammatory drug (NSAID) with analgesic, anti-inflammatory and antipyretic properties. These properties are primarily achieved by its ability to block the enzyme cyclooxygenase, but also by an additional direct effect on hyperalgesia due to the functional down-regulation of sensitised peripheral pain receptors [1]. The efficacy of diclofenac equals that of many newer and established NSAIDs. As an analgesic it has a fast onset and long duration of action. Compared to other NSAIDs, diclofenac is well tolerated and rarely produces gastrointestinal ulcerations or other serious side-effects. Thus, diclofenac can be considered as one of few non-steroidal anti-inflammatory drugs of first choice used in the treatment of acute and chronic painful and inflammatory conditions [2].

Several methods for the determination of diclofenac in biological fluids have been reported. They include gas chromatography with electron capture detection [3,4], gas chromatography–mass spectrometry [5] and high-performance liquid chromatography using either ultraviolet [6–14], fluorescence [15,16] or electrochemical [17,18] detection. HPLC with ultraviolet detection is the most popular method for quantification of diclofenac. Nevertheless these methods have some shortcomings—the major one is a lack of sensitivity, while another one is poor specificity—and therefore they are liable to interference from diclofenac metabolites or other endogenous compounds. Multiple extraction steps can also be involved in these methods. In contrast to HPLC, the gas chromatography methods possess adequate sensitivity and specificity, but they require large sample volume, time-consuming derivatization and sample

*Corresponding author. Tel.: +386-1-4769-500; fax: +386-1-4258-031.
E-mail address: robert.roskar@ffa.uni-lj.si (R. Roškar).

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clean-up procedures, as well as complex instrumentation.

In this paper we describe a simple and specific method for quantitative determination of diclofenac in synovial fluid. We used a single liquid–liquid extraction step characterised by good and reproducible recovery as a clean-up procedure. This was followed by a rapid and sensitive HPLC assay. Analyses of the synovial samples taken from patients treated with diclofenac proved the developed method to be suitable for monitoring the concentrations of diclofenac in synovial fluid.

2. Experimental

2.1. Materials

Diclofenac sodium was supplied by Krka (Slovenia), human albumin by Octapharma (Austria) and mucin by Sigma (UK). All solvents and chemicals were supplied by Merck (Darmstadt, Germany). Acetonitrile and methanol were of HPLC grade, and all other reagents were of analytical grade.

2.2. Chromatography

We used an HP 1100 high-performance liquid chromatograph controlled by Chemstation, equipped with a thermostated autosampler, column heater and diode array detector. The flow rate was set at 1 ml/min, the volume of injection at 100 μl and UV detection for monitoring at the wavelength of 205 nm. A Kromasil C_{18} column (5 μm; 150×4.6 mm) with an inline filter was used. The column was thermostated at 35 °C. The mobile phase consisted of 0.05 *M* KH_2PO_4 aqueous solution (adjusted to pH 7 with NaOH) and methanol and acetonitrile in the ratio of 58:21:21 (v/v/v).

2.3. Preparation of standard solutions

2.3.1. Standard solutions of diclofenac in water

A standard stock solution of diclofenac sodium was prepared in bidistilled water with a concentration of 10 μg/ml. A series of standard solutions at concentrations of 30, 45, 100, 300 and 500 ng/ml were prepared by further dilution of the standard stock solution in bidistilled water.

2.3.2. Standard solutions of diclofenac in drug free synovial fluid

Synovial fluid standard solutions were prepared by spiking 200 μl of diclofenac water standard solutions into 1 ml of drug free synovial fluid. By using water solutions of different drug content, a series of synovial fluid standards was prepared at drug concentrations as above.

2.3.3. Standard solution of diclofenac in albumin/mucin solution

The solution of albumin (45 mg/ml) and mucin (1.2 mg/ml) was prepared by mixing 20% human albumin solution with powdered mucin and bidistilled water. Then 1 ml of this solution was spiked with 200 μl diclofenac water standard solution to achieve the standard solution of diclofenac with a concentration of 300 ng/ml.

2.4. Sample preparation

A 2-ml sample of orthophosphoric acid (0.83 *M*) was added to 1.2 ml of the synovial fluid sample and vortexed for several seconds. Then 0.6 ml of isopropylalcohol was added and vortexed again for several seconds. After adding 5.4 ml of *n*-hexane, the mixture was vortexed for 1 min and then centrifuged at 5000 rpm for 5 min. The organic phase was transferred into a clean tube and the solvent evaporated at 30 °C under nitrogen flow. The residue was dissolved in 1.2 ml of the mobile phase by vortexing. An aliquot of 100 μl was injected into the liquid chromatograph. The same procedure was used for albumin/mucin solutions in preliminary recovery determinations.

2.5. Recovery determination

The recovery of diclofenac was evaluated by comparing the peak areas from the extracted diclofenac standard solutions in the drug free synovial fluid to the areas obtained from the unextracted standard solutions of diclofenac in water. The preliminary recoveries based on albumin/mucin solutions determined during optimisation of the method

were obtained in the same way as those for true synovial fluids described above.

3. Results and discussion

3.1. Chromatography and sample preparation

Synovial fluid, the effusion that is usually aspirated from the knee of a rheumatoid arthritis patient, has a high protein and fat content compared to plasma or urine. This medium also contains large amounts of a glycosaminoglycan hyaluronic acid [19]. The differences in the composition of biological fluids usually require different approaches to the preparation of a sample. In most cases, however, the clean-up procedures for synovial and plasma samples are identical to those reported in the literature. The most commonly applied clean-up techniques for biological samples are direct injection, protein precipitation, liquid–liquid extraction and solid-phase extraction [20,21]. Each procedure has some advantages but also some drawbacks concerning different biological samples. On the one hand the direct injection technique can in the long-run cause the chromatographic column to deteriorate. This can also occur with sample injections after protein precipitation. On the other hand solid-phase extraction can be quite time-consuming when trying to establish optimal experimental conditions. Therefore we chose a simple one step liquid–liquid extraction based on literature data and then upgraded it to achieve better reproducibility and accuracy.

We began our study with a procedure used for diclofenac determination in the plasma as well as in the articular cartilage, synovial membrane and bones [14]. The samples were prepared by protein precipitation and subsequent extraction with a mixture of *n*-hexane and isopropylalcohol. This method gave good recoveries, yet in some cases a gel was formed throughout the organic phase. Other approaches were then taken into consideration. Since we achieved good recoveries, we kept the liquid–liquid extraction but modified it so as to prevent gel formation without affecting the recovery. For this purpose we changed the main variables of the method, i.e. the extraction solvent, the strength of the acid for protein precipitation, the duration of shaking and the volume ratio between the samples and the solvents. Large volumes of the relevant blank biological fluid were not available therefore the albumin/mucin solution was used instead of synovial fluid during optimisation of the method. The solution had similar composition with regard to the concentrations of albumins and glycosaminoglycans as the synovial fluid and this made it suitable for determination of preliminary recoveries as described in Section 2.5. So the best results for the clean-up procedure were obtained by adding isopropylalcohol to the albumin/mucin solution in the first step, followed by extraction of diclofenac with *n*-hexane (Table 1) as described in the Experimental section. It is assumed that by this procedure the transfer of water molecules into organic layer was hindered, simultaneously preventing gel formation in the organic phase.

Following the clean-up procedure, chromatographic separation of the prepared samples was achieved by reversed-phase liquid chromatography. A wavelength of 205 nm was chosen to increase the sensitivity of diclofenac detection although the interferences at this wavelength were slightly bigger than those observed at 282 nm, which was the wavelength

Table 1
Estimated recoveries of diclofenac obtained from albumin/mucin solutions using different extraction solvent combinations, each based on six replicates

Sample	Extraction solvent	Gel formation	Recovery (%)
AM	*n*-Hexane	No	Insufficient
AM	*n*-Hexane/IPA (9:1)	Yes	70
AM	*n*-Hexane/acetone (9:1)	Yes	60
AM + acetone	*n*-Hexane	No	80
AM + IPA	*n*-Hexane	No	90

AM, albumin/mucin solution; IPA, isopropylalcohol.

chosen previously [14]. The resolution was not satisfactory when the mobile phase consisted of 0.05 *M* KH_2PO_4 aqueous solution (adjusted to pH 7) and methanol. The retention time of diclofenac was prolonged by increasing the percent of buffer in the mobile phase to obtain better resolution. In this case the increase of backpressure required the replacement of methanol with a mixture of methanol and acetonitrile (1:1, v/v). Such optimisation of the mobile phase resulted in well-shaped peaks of diclofenac with retention times of 11 min with good resolution. Typical chromatograms of blank synovial fluid, of standard diclofenac solution and of synovial fluid spiked with diclofenac are shown in Figs. 1–3.

3.2. Linearity and sensitivity

Calibration curves were prepared by analysing standard solutions of diclofenac in drug free synovial fluid. Three calibration curves based on five concentration points (three replicates) for each curve were analysed on three subsequent days. They showed good linear relationship obtained by the least square linear regression model within the concentration range of 30–500 ng/ml. The obtained determination coefficients (r^2) exceeded 0.99 and the equation based on all data was y (area) = 6.42 + 0.59**c* (ng/ml). Quantification and detection limits were estimated according to ICH guidelines using average slope estimation and the standard deviation of y-intercepts of regression lines [22]. The values were 45 and 15 ng/ml for LOQ and LOD, respectively. The limit could be further lowered by a suitable modification of the sample preparation. We observed that the residue could also be reconstituted with 600 or 400 μl of mobile phase, lowering the limits accordingly. In this volume range the correlation between the volume used and the response of

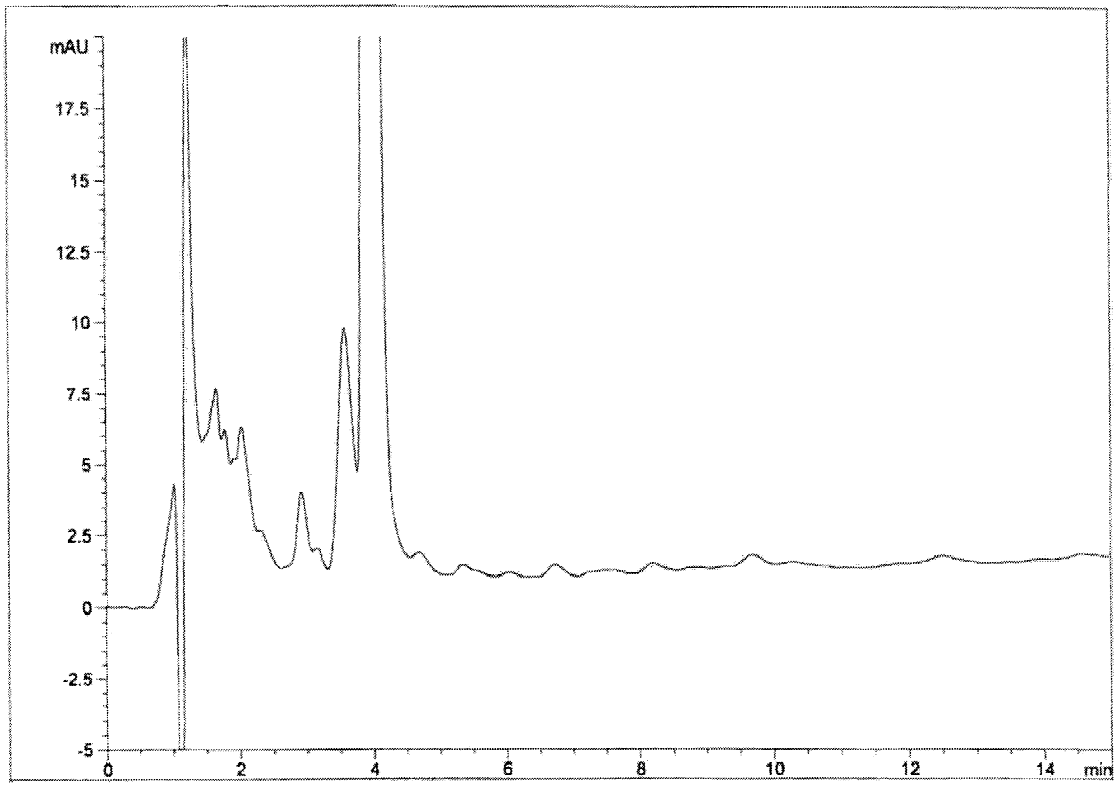

Fig. 1. Chromatogram of blank synovial fluid.

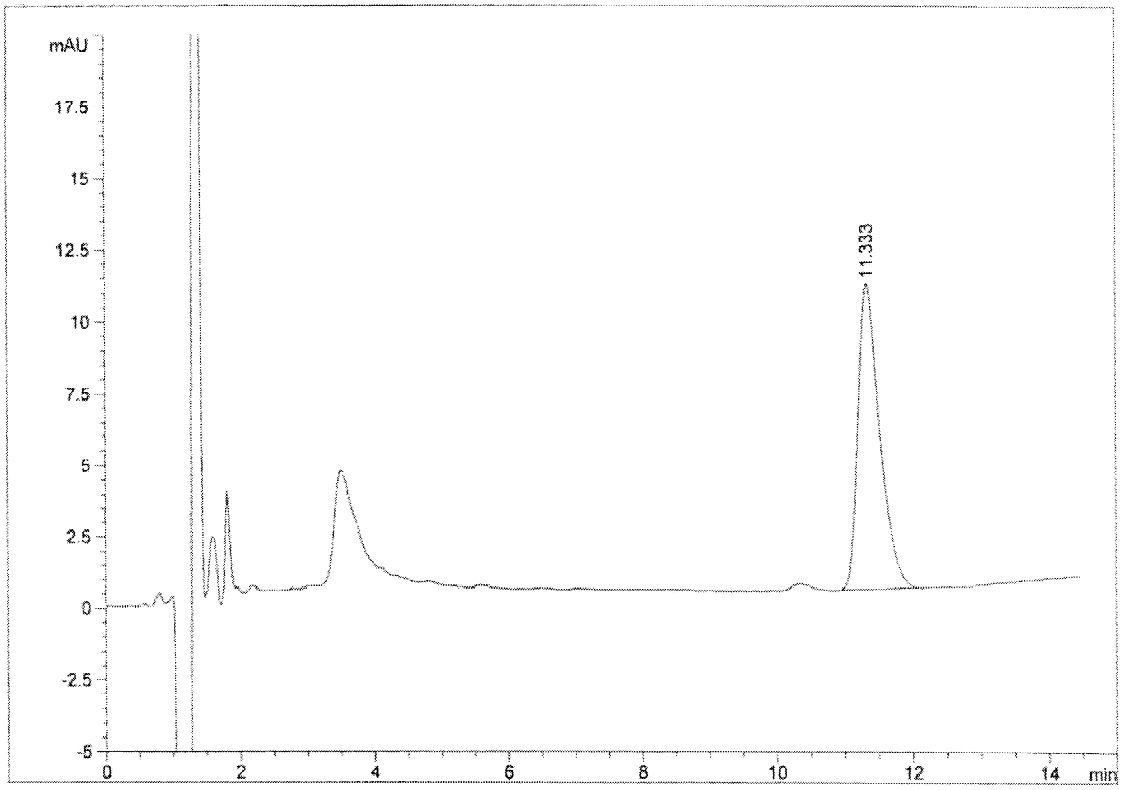

Fig. 2. Chromatogram of standard diclofenac solution (300 ng/ml).

the signal was still linear. By further lowering the solvent volume, the interferences between chromatographic peaks became more expressed.

3.3. Accuracy and precision

Accuracy was determined by comparing concentrations calculated from calibration curve and known concentrations of spiked synovial standard solutions. On an intra-day basis an average accuracy of 97–106% was found, except for the lowest concentration where the accuracy was 88%. The inter-day accuracy was 94–107% for higher concentrations, and 84% for the lowest one (Table 2).

Precision expressed as coefficient of variation (C.V.) was below 6.2% for the calibration range, except for the lowest concentration with C.V. of 14.5%. Intra-day values were within the range of 2.3–8.3% (Table 2).

3.4. Recovery

As shown in Table 3, the recovery of diclofenac was above 87%, with a mean value of 91% confirming the preliminary results based on albumin/mucin solutions. The recoveries were consistent for the samples as demonstrated by SD of less than 5.0%. Recovery did not depend on the concentration, which resulted in good linearity of the calibration curve. Good results obtained by the described method convinced us that there was no need to introduce an internal standard approach for subsequent analyses of the synovial samples.

3.5. Assay application

Applicability of this method was proven by analysing true samples obtained after administration of 50 mg diclofenac sodium to patients affected by

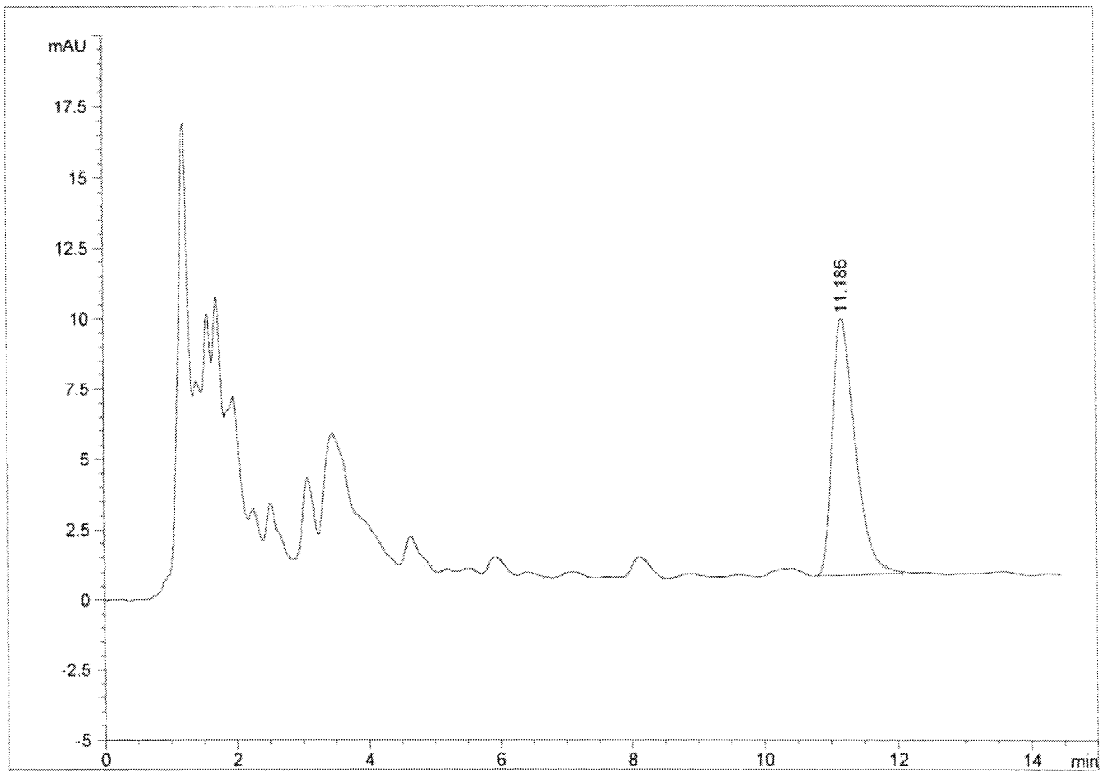

Fig. 3. Chromatogram of synovial fluid spiked with diclofenac (300 ng/ml).

rheumatoid arthritis. A typical chromatogram of a true sample is shown in Fig. 4. No interferences from endogenous compounds or metabolites of diclofenac were found. Although the sensitivity of this method is lower than that found with GC–MS or HPLC with electrochemical detection, it is sufficient for determination of the expected diclofenac concentration in synovial fluid [23,24]. Furthermore, this method is equally or even more sensitive than other reported HPLC methods with ultraviolet detection used for determination of diclofenac in the plasma samples.

Table 2
Accuracy and precision of diclofenac assay

Concentration spiked (ng/ml)	Concentration found (ng/ml)	% of Spiked concentration	C.V. (%)
Intra-day (n = 3)			
30	26.5	88	8.3
45	44.3	98	3.1
100	105.0	105	3.4
300	317.0	106	5.3
500	482.5	97	2.3
Inter-day (n = 3)			
30	26.7	84	14.5
45	42.4	94	6.2
100	100.6	101	3.6
300	321.6	107	2.9
500	484.3	97	4.1

4. Conclusions

The present method for determination of diclofenac in synovial fluid is simple and rapid with a single extraction step. No derivatization or complex instrumentation is needed. Data obtained by this approach show that the method is reliable and suitable for monitoring diclofenac levels in synovial fluid after oral or cutaneous administration of the drug to patients with inflammatory and degenerative joint diseases.

Table 3
Recovery of diclofenac from synovial fluid ($n=3$)

Concentration (ng/ml)	Peak area (mAU)		Recovery (%)	SD (%)
	Standard solution	Synovial fluid		
45	33.3	31.4	94.3	2.9
100	74.8	65.7	87.8	3.0
300	212.6	199.4	93.8	4.9
500	354.0	308.2	87.0	4.3
		Mean:	90.7	

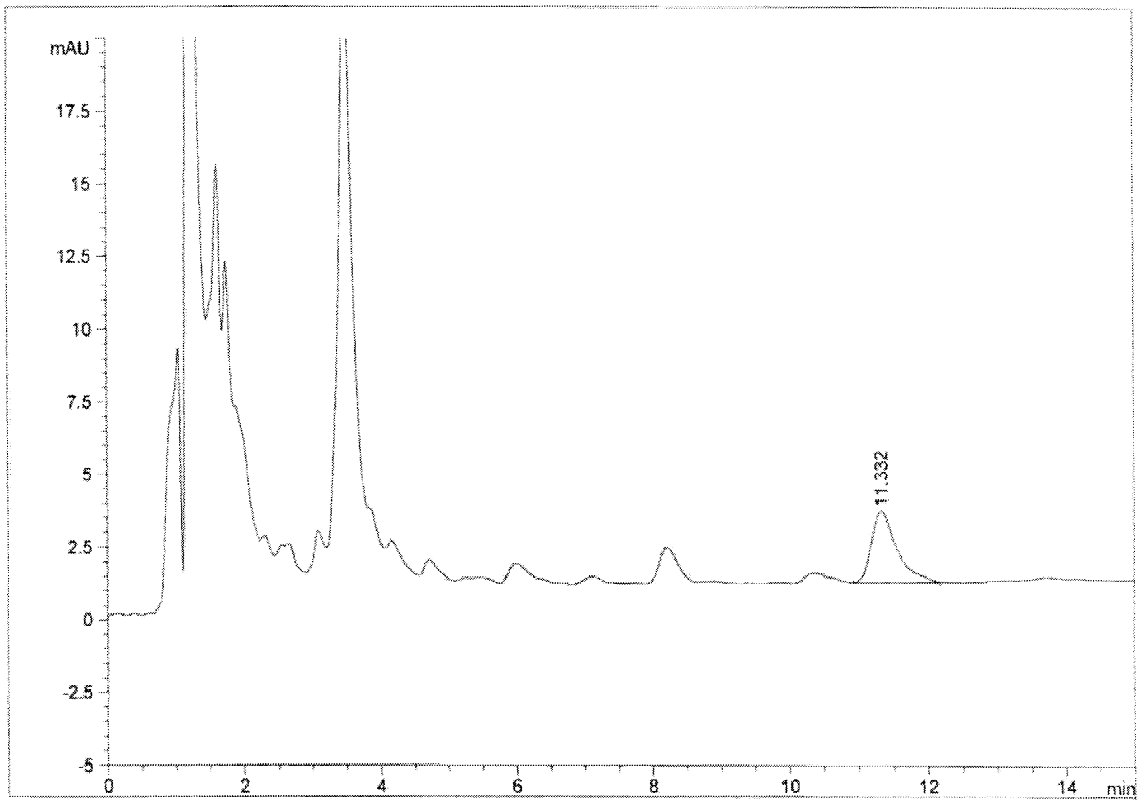

Fig. 4. Chromatogram of synovial fluid sample from a patient with rheumatoid arthritis following oral administration of diclofenac.

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