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Note

High-performance liquid chromatographic determination of 6-desmethylnaproxen sulfate in human plasma

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Naproxen is a non-steroidal anti-inflammatory agent that is used in the treatment of rheumatoid arthritis and osteoarthritis and as an analgesic. Recently we presented evidence for the presence in plasma of 6-desmethylnaproxen sulfate (6-DMNS) as a metabolite of naproxen and demonstrated that the concentration is elevated in patients with impaired renal function [1]. The evidence for the presence of 6-DMNS in plasma and the method employed for the quantification of 6-DMNS involved the determination of 6-desmethylnaproxen (6-DMN) in plasma before and after incubation of the plasma with sulfatase. In this paper we describe a much simpler method for the determination of 6-DMNS in plasma. The method involves precipitation of plasma proteins using a mixture of acetonitrile—methanol (2:1) followed by anion-exchange high-performance liquid chromatography (HPLC) with fluorescence detection and quantification.

EXPERIMENTAL

Chemicals and reagents

Acetonitrile and methanol, HPLC grade, were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.); glacial acetic acid from Mallinckrodt (McCraw Park, IL, U.S.A.); ammonium phosphate, monobasic, from Mallinckrodt (St. Louis, MO, U.S.A.); sodium pyrophosphate from Matheson Coleman and Bell (East Rutherford, NJ, U.S.A.); tetrabutylammonium phosphate from Kodak (Rochester, NY, U.S.A.) and PIC® A was from Waters Assoc. (Milford, MA,

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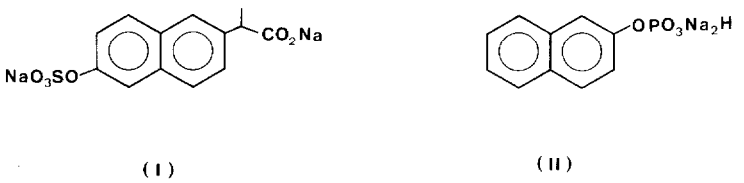

(I) (II)

Fig. 1. Chemical structures of 6-desmethylnaproxen sulfate disodium salt (I) and 2-naphthyl phosphate disodium salt dehydrate (II).

U.S.A.). 6-Desmethylnaproxen sulfate disodium salt (I) and 2-naphthyl phosphate disodium salt dehydrate (II) (Fig. 1) were obtained from the Institute of Organic Chemistry (Syntex Research, Palo Alto, CA, U.S.A.). Water was purified by means of a Milli-Q System (Millipore, Bedford, MA, U.S.A.).

Preparation of stock and spiked solutions of 6-DMNS and internal standard (2-naphthyl phosphate)

Stock solutions of 6-DMNS at concentrations of 100, 10 and 1 μg/ml were prepared in methanol. From the stock solutions the following spiked solutions at concentrations of 2000, 1000, 500, 300, 200, 100, 50, 25 and 10 ng/ml were prepared in a mixture of methanol—water (1:1). The spiked solution of internal standard at a concentration of 2 μg/ml also was prepared in a mixture of methanol—water (1:1).

Preparation of samples of plasma for analysis

Into a 15-ml test tube were added sequentially, plasma (0.1—1.0 ml), 200 μl of saturated sodium pyrophosphate solution and 500 μl of internal standard spiked solution, with brief vortex-mixing after the second and third additions. To the final solution were added 3 ml of a mixture of acetonitrile–methanol (2:1) and the contents were vortex-mixed for 5 s to precipitate the proteins. After centrifugation for 5 min at 280 *g*, 2 ml of the supernatant were transferred into an HPLC injection vial containing 1 ml of water. The same procedure was employed for calibration standards after 500 μl of 6-DMNS spiked solutions over the desired concentration ranges were added to 1 ml of control plasma.

HPLC analysis

A Whatman 10-μm SAX column (25 cm × 4.6 mm) was employed for the analysis. The mobile phase was a mixture of 40.5% methanol in 0.01 *M* tetrabutylammonium phosphate solution previously adjusted to pH 7.10 with saturated ammonium phosphate, monobasic. The analytes in 50—100 μl of the diluted supernatants were injected onto the column with a Waters 710B autosampler and eluted with the mobile phase at a flow-rate of 1.0 ml/min by means of a Waters 6000A solvent delivery system. The eluates were monitored with a Schoeffel FS970LC fluorescence detector equipped with a deuterium lamp and an autoreset device. The chromatograms were recorded on a Houston strip chart recorder. The HPLC system was operated at ambient temperature.

RESULTS AND DISCUSSION

At an organic solvent-to-plasma ratio of 3:1, the solvent mixture consisting of two parts of acetonitrile and one part of methanol precipitates more than 99% of the proteins in plasma [2]. After centrifugation, a clear supernatant containing more than 99% of the added 6-DMNS in 0.1–1.0 ml of blank plasma is obtained which can be injected directly onto the HPLC column. Precipitation with 100% acetonitrile at the same ratio of solvent to plasma

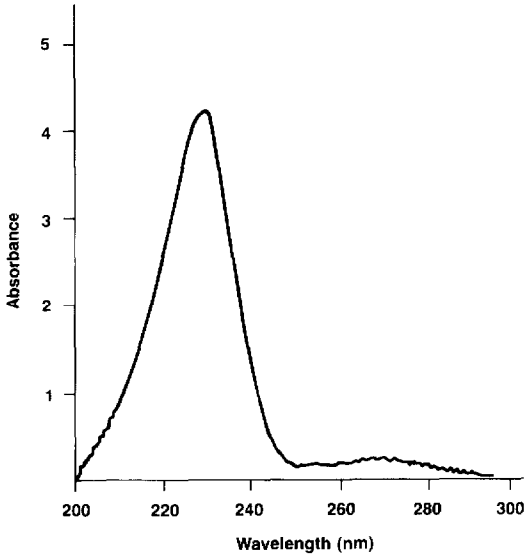

Fig. 2. Corrected excitation spectrum for 6-DMNS measured with a Schoeffel 970 fluorescence detector equipped with a 320-nm cut-off emission filter, a deuterium lamp, a memory module (MM701) and a wavelength drive (SFA339).

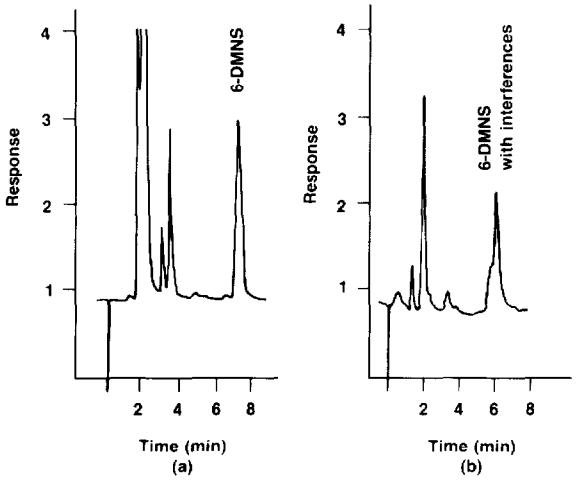

Fig. 3. HPLC profiles of extracts of 1 ml of plasma spiked with 6-DMNS chromatographed on (a) a strong anion-exchange column (SAX, 10 μm, 25 cm × 4.6 mm); (b) a reversed-phase C_{18} column (5 μm, 25 cm × 4.6 mm). Mobile phase: acetonitrile—methanol—PIC A (12:10:78).

yielded a low recovery (70%) of the added 6-DMNS although the same precipitation procedure yielded much higher recoveries (90—95%) for naproxen and 6-DMN [3, 4]. The difference in recovery may be a result of the lower solubility of 6-DMNS in acetonitrile. Precipitation with 100% methanol yielded a cloudy supernatant which was not suitable for direct injection onto the HPLC column.

Similar to naproxen or 6-DMN, 6-DMNS has strong inherent fluorescence. For HPLC analysis of 6-DMNS with a fluorescence detector equipped with a deuterium lamp, the maximum sensitivity was obtained with an excitation wavelength of 229 nm and an emission cut-off filter of 320 nm. (Fig. 2).

A comparison of ion-exchange and reversed-phase columns for the separation of 6-DMNS that had been spiked into plasma is shown in Fig. 3a and b, respectively. Clearly, only the ion-exchange column provides a suitable separation. The chromatographic system employed in the method described here provided satisfactory results in terms of separation, retention characteristics and peak shapes of 6-DMNS and internal standard (Fig. 4).

Assay of blank plasma from normal or uremic subjects or from subjects who had previously received 325 mg of aspirin and 60 mg of codeine phosphate or 300 mg of acetaminophen and 60 mg of codeine phosphate revealed no interfering peaks in the elution profiles for 6-DMNS or internal standard (Fig. 5).

2-Naphthyl phosphate was selected as the internal standard because of its close similarity in HPLC characteristics to those of 6-DMNS. Hydrolysis of the phosphate during the extraction process by phosphatases prsent in plasma was prevented by the addition of an excess of sodium pyrophosphate, a competitive inhibitor of phosphatase.

Based on an acceptable signal-to-noise ratio of 10:1 the limit of detection of 6-DMNS is 10 ng/ml of plasma (Fig. 4b). The method is linear and reproducible

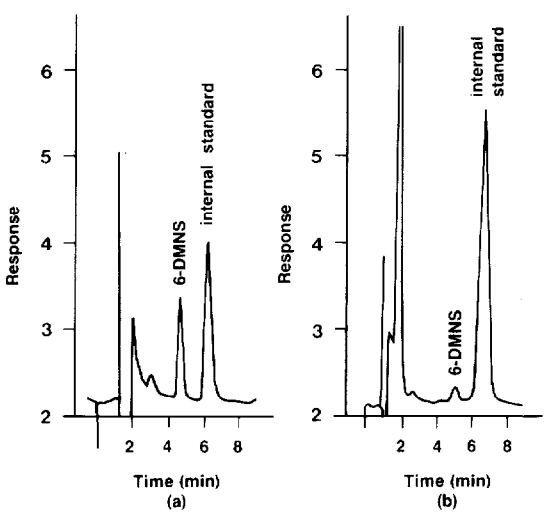

Fig. 4. HPLC profiles of extracts of (a) 1 ml of plasma from a subject previously treated with naproxen; 1 μg of internal standard, 2-naphthyl phosphate, was added prior to extraction; (b) 1 ml of control plasma spiked with 10 ng of 6-DMNS and 1 μg of internal standard.

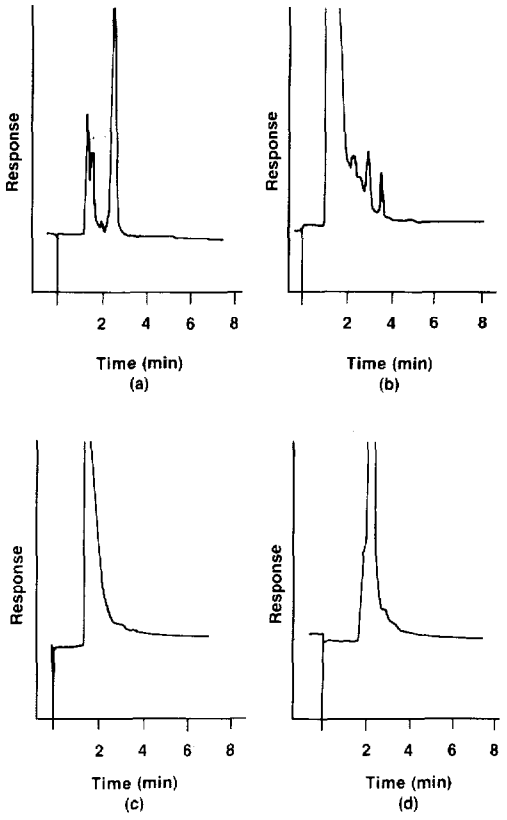

Fig. 5. HPLC profiles of extracts of 1 ml of plasma from: (a) a normal subject, immediately before treatment with naproxen; (b) a uremic patient, immediately before treatment with naproxen; (c) an orthopedic patient, 2 h after administration of 325 mg of aspirin and 60 mg of codeine phosphate; (d) an orthopedic patient, 2 h after administration of 300 mg of acetaminophen and 60 mg of codeine phosphate.

TABLE I

ANALYSIS OF THE DATA FOR THREE CALIBRATION CURVES FOR THE ASSAY OF 6-DMNS OVER THE RANGE 10—500 ng/ml IN PLASMA

Quantity spiked (ng/ml)	Mean of three determinations (ng/ml)	Coefficient of variation (%)
0	0	–
10	11.6	4.7
20	22.0	7.1
30	31.3	1.0
50	49.5	1.2
100	97.5	2.5
200	196.7	2.2
300	291.4	0.9
500	506.9	0.2
y-Intercept	0.02475	19.4
Slope	0.00770	1.4
Correlation coefficient (*r*)	0.9996	0

TABLE II

ANALYSIS OF THE DATA FOR THREE CALIBRATION CURVES FOR THE ASSAY OF 6-DMNS OVER THE RANGE 100—2000 ng/ml IN PLASMA

Quantity spiked (ng/ml)	Mean of three determinations (ng/ml)	Coefficient of variation (%)
0	0	-
100	104.9	1.3
200	196.6	1.4
300	290.0	2.2
500	485.8	3.4
1000	968.7	2.4
2000	2020.5	0.6
y-Intercept	−8.4112	44.7
Slope	1.0063	0.3
Correlation coefficient (r)	0.9996	0.03

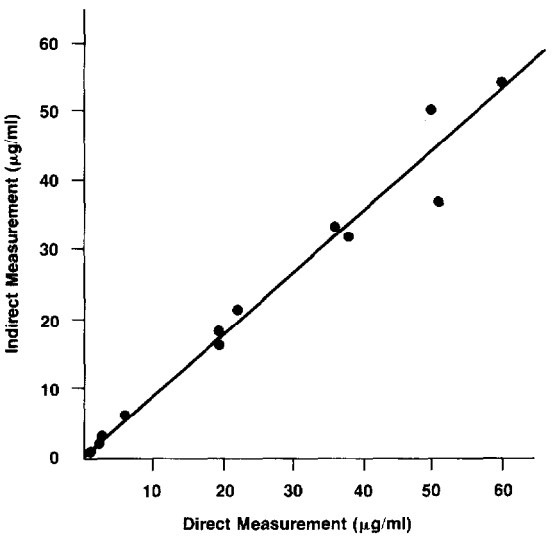

Fig. 6. Comparison of results of determination of 6-DMNS in plasma from naproxen-treated uremic subjects: direct assay versus indirect assay involving determination of 6-DMN before and after incubation of the plasma with sulfatase. $y = 0.8808x + 0.1000$; $r = 0.9898$.

over the calibration ranges 10—500 and 100—2000 ng. Analysis in triplicate of blank plasma samples spiked with 6-DMNS over the range 10—500 ng/ml yielded an average coefficient of variation (C.V.) of 2.5%, $r = 0.9996$ (Table I) and over the range of 100—2000 ng/ml yielded an average C.V. of 1.9%, $r = 0.9996$ (Table II).

Selected samples of plasma from normal and uremic subjects who had been treated with naproxen were assayed using this method. The results are in good agreement with those obtained previously using a more laborious method that involves HPLC determination of 6-DMN before and after incubation of the plasma with sulfatase [1]. The following linear regression equation was calculated; $y = 0.8808x + 0.1000$; $r = 0.9898$ (Fig. 6).

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