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Note

Simultaneous determination of nimesulide and hydroxynimesulide in human plasma and urine by high-performance liquid chromatography

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Nimesulide [4-nitro-2-(phenoxy)methanesulphonanilide] is a drug with anti-inflammatory activity [1]. As the therapeutic doses (50–100 mg twice daily) are relatively low, a sensitive and specific method is required to study the pharmacokinetics of the drug. Although a high-performance liquid chromatographic method has already been reported [2], a new method has been developed in order to obtain greater sensitivity and to perform simultaneous determinations of the levels of nimesulide and its major metabolite hydroxynimesulide [4-nitro-2-(4′-hydroxyphenoxy)methanesulphonanilide].

EXPERIMENTAL

Chemicals

All reagents were of analytical grade unless indicated otherwise.

Potassium dihydrogenphosphate, hydrochloric acid, methanol of HPLC grade (Carlo Erba, Milan Italy) and benzene (Merck, Darmstadt, F.R.G.) were used. Nimesulide, hydroxynimesulide and tolbutamide were supplied by BBR (Milan, Italy). β-Glucuronidase (20 U/mg at 37°C, BBR) was used for the hydrolysis of conjugated metabolites for the assay of total hydroxynimesulide.

Chromatographic system

An isocratic HPLC system was used, consisting of a Model 510 pump (Waters Assoc., Milford, MA, U.S.A.), a Model 490 programmable multi-wavelength detector (Waters Assoc.) focused at 230 nm and a Waters 840 chromatographic control station. A C_{18} reversed-phase column (30 cm × 4.6 mm I.D., 10 μm particle size, Merck) was employed. The samples were injected through a WISP

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710B automatic sampler (Waters Assoc.). The mobile phase was 0.05 *M* phosphate buffer (pH 5.0)–methanol (50:50, v/v). Chromatography was carried out at a flow-rate of 1.0 ml/min at room temperature (25°C).

Preparation of solutions

Concentrated standard solutions containing nimesulide and hydroxynimesulide were prepared by dissolving 10 mg of each substance in 10 ml of methanol. An internal standard stock solution was prepared by dissolving and diluting tolbutamide with methanol to 2.5 μg per 10 μl.

Extraction procedure

Nimesulide and unconjugated hydroxynimesulide. Nimesulide and hydroxynimesulide were extracted from 1-ml plasma or urine samples spiked with 10 μl of internal standard solution. After acidification with 10 μl of concentrated hydrochloric acid (pH adjusted to 1), 8 ml of benzene* were added and the samples were extracted twice for 15 min. After centrifugation (1500 *g* for 10 min) the organic layer was dried under a flow of nitrogen at room temperature. The residue was dissolved in 200 μl of the mobile phase and the solution transferred into the vials of the automatic sampler. Volumes of 5 μl were injected into the chromatograph and the assay was conducted as described.

Nimesulide and total (free and conjugated) hydroxynimesulide. Urine samples (2 ml) were adjusted to pH 5.5 with 50 m*M* sodium acetate buffer and 0.2 ml of β-glucuronidase solution (200 U/ml at 37°C) was added. After incubation at 37°C for 3 h, 10 μl of concentrated hydrochloric acid and 20 μl of internal standard solution were added. The mixture was extracted twice with 8 ml of benzene and the analysis performed as described.

Plasma and urine calibration graphs

Calibration graphs were obtained by assaying samples of blank plasma and urine added with appropriate amounts of nimesulide, hydroxynimesulide and internal standard stock solution. The internal standard concentration was 2.5 μg/ml for each plasma sample. Tables I and II show the ratios of the nimesulide and hydroxynimesulide peak areas to that of the internal standard. The detector response was linear in the ranges investigated. The parameters for the regression curves are also reported.

RESULTS

Typical chromatograms for plasma and urine samples are shown in Fig. 1. Under the experimental conditions used no interfering peaks appear after the injection of samples corresponding to plasma and urine from untreated subjects. A complete separation between nimesulide, hydroxynimesulide and the internal standard was achieved.

*Instead of benzene also toluene can be used.

TABLE I

NIMESULIDE (A) AND HYDROXYNIMESULIDE (B) CALIBRATION GRAPHS FOR THE PLASMA ASSAY

The regression curves were constructed by linear regression fitting and their mathematical expressions are $y = -0.01708 + 0.3034x$ ($r = 0.9999$) for A and $y = 0.007029 + 0.30677x$ ($r = 0.9999$) for B, where x = concentation of injected samples and y = nimesulide or hydroxynimesulide peak area/internal standard (I.S.) peak area ($n = 5$).

Concentration added (μg/ml)		Mean compound/I.S. peak-area ratio		Coefficient of variation (%)	
A	B	A	B	A	B
0.25	0.10	0.0574	0.0350	1.99	1.31
0.50	0.25	0.1360	0.0848	0.74	0.94
1.00	0.50	0.2888	0.1656	0.75	0.92
2.50	1.00	0.7376	0.3096	0.26	0.84
5.00	2.50	1.5013	0.7746	0.18	0.65

TABLE II

NIMESULIDE (A) AND HYDROXYNIMESULIDE (B) CALIBRATION GRAPHS FOR THE URINE ASSAY

The regression curves were constructed by linear regression fitting and their mathematical expressions are $y = -0.00545 + 0.2504x$ ($r = 0.9999$) for A and $y = -0.00965 + 0.3184x$ ($r = 0.9999$) for B (symbols as in Table I); $n = 5$.

Concentration added (μg/ml)		Mean compound/I.S. peak-area ratio		Coefficient of variation (%)	
A	B	A	B	A	B
0.05	1.00	0.0070	0.3088	2.75	0.53
0.10	5.00	0.0197	1.5827	1.42	0.10
0.20	10.00	0.0446	3.1750	0.75	0.09

Recovery

Recoveries from human plasma and urine were determined in duplicate by comparing nimesulide and hydroxynimesulide peak areas after injection of standard solutions and after the extraction of plasma and urine samples at the same concentrations employed for the calibration graphs. Nimesulide and hydroxynimesulide were quantitatively extracted from plasma (93 and 88%) and urine (98 and 92%); the recovery did not depend on the amounts present in the specimens.

Limit of detection

The minimum detectable concentrations of nimesulide and hydroxynimesulide were 50 ng/ml for both plasma and urine.

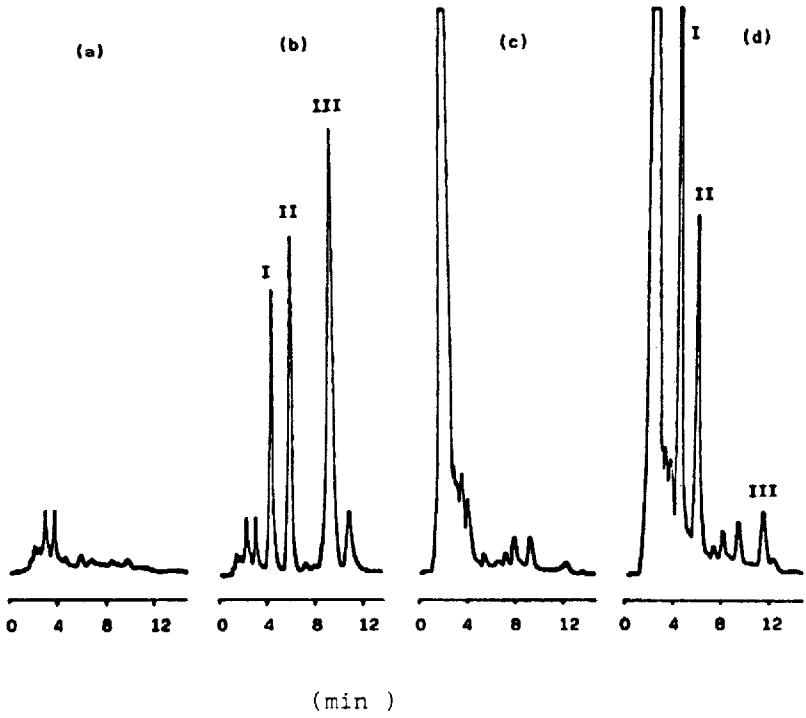

Fig. 1. Chromatograms of human plasma and urine extracts. Plasma: (a) specimen from an untreated subject; (b) specimen from a subject treated with nimesulide (nimesulide found 4.98 μg/ml; hydroxynimesulide found, 2.08 μg/ml), spiked with tolbutamide (see text). Urine: (c) specimen from an untreated subject; (d) specimen from a subject treated with nimesulide (nimesulide found, 0.68 μg/ml; hydroxynimesulide found, 5.00 μg/ml) spiked with tolbutamide (see text). Peaks: I = hydroxynimesulide; II = tolbutamide; III = nimesulide.

Accuracy and precision

The accuracy and precision of the method were checked by determining plasma levels in samples obtained by adding appropriate amounts of the standard solution of hydroxynimesulide and nimesulide to blank plasma. The same procedure was carried out for urine samples. Tables III and IV give the results, which indicate that both the accuracy and precision were excellent.

Drug interferences

In order to evaluate possible interferences, some drugs were tested at the same analytical conditions as employed for nimesulide. All drugs were tested at therapeutic concentrations, known from the current literature. It was found that digoxin, flurazepam and tiadenol do not interfere and do not give a detector response; glibenclamide, theophylline, doxepin, paracetamol, salicylic acid and acetylsalicylic acid do not interfere but give a detector response. Bezafibrate shows the same retention time as tolbutamide and hence interferes in the determination of nimesulide and hydroxynimesulide by the proposed method.

TABLE III

ACCURACY AND PRECISION OF THE DETERMINATION OF NIMESULIDE (A) AND HYDROXYNIMESULIDE (B) IN HUMAN PLASMA ($n=5$)

Concentration added (μg/ml)		Concentration found (μg/ml)		Coefficient of variation (%)	
A	B	A	B	A	B
0.25	0.10	0.245	0.098	3.8	2.1
0.75	0.75	0.747	0.766	2.1	2.2
2.50	1.00	2.519	0.991	1.9	1.9
5.00	2.50	4.980	2.581	0.7	1.8

TABLE IV

ACCURACY AND PRECISION OF THE DETERMINATION OF NIMESULIDE (A) AND HYDROXYNIMESULIDE (B) IN HUMAN URINE ($n=5$)

Concentration added (μg/ml)		Concentration found (μg/ml)		Coefficient of variation (%)	
A	B	A	B	A	B
0.05	1.00	0.048	0.989	3.9	2.2
0.10	5.00	0.097	5.230	2.8	1.2
0.20	10.00	0.188	10.116	2.2	0.9

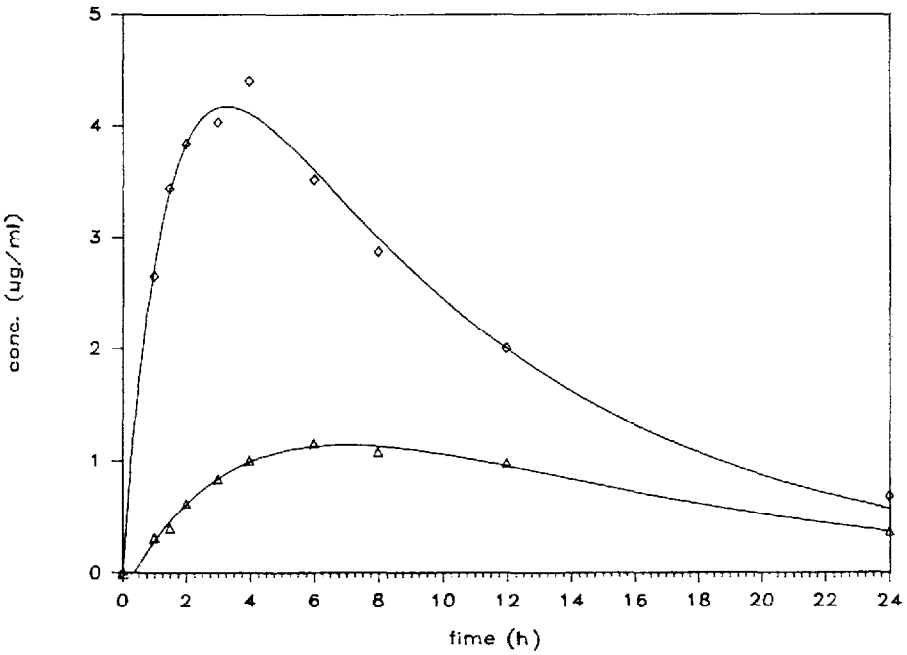

Fig. 2. Computer-generated nimesulide and hydroxynimesulide mean plasma levels and experimentally obtained values (◇, nimesulide; △, hydroxynimesulide).

Nimesulide and hydroxynimesulide plasma levels and urinary excretion

Five healthy volunteers aged from 52 to 65 years (mean 63 years) received one tablet containing 100 mg of nimesulide (Aulin, BBR) at 8.00 a.m. All subjects had fasted since the previous night and no food was allowed for 4 h after drug administration. Serial samples of blood and urine were collected during the 24 h after administration. Plasma and urine specimens were kept frozen at −20°C until analysed.

Fig. 2 shows the time course of the mean plasma levels of nimesulide and free hydroxynimesulide; conjugated hydroxynimesulide was not detectable. These data were also fitted to bi-exponential equations using the NONLIN–AUTOAN procedure [3, 4] in order to obtain a quantitative description of nimesulide disposition. After hydrolysis of plasma samples the hydroxynimesulide concentration did not increase. The urinary excretion of the unchanged drug was found to be less than 0.07% of the administered dose within 24 h. The average values for free and conjugated hydroxynimesulide were 0.98 and 23.19%.

DISCUSSION

The aim of this study was to develop a direct assay for determining simultaneously plasma and urine levels of nimesulide and hydroxynimesulide. The proposed method allows an excellent separation of nimesulide and its metabolite. The sensitivity (50 ng/ml for both nimesulide and hydroxynimesulide) is adequate for determining their plasma and urine concentrations after therapeutic doses of nimesulide. Both the precision and accuracy are satisfactory. Further, the rapidity of the analytical procedure makes it possible to analyse 40 samples of plasma or urine within 24 h. Another advantage is that a readily available internal standard is used. A pilot study was carried out on volunteers in order to demonstrate the validity of the assay method itself. The results demonstrate the possibility of determining nimesulide and hydroxynimesulide levels in order to investigate their pharmacokinetics in man. In terms of nimesulide plasma and urine levels, our results are in agreement with previously published data [5].

REFERENCES

1 T.J. Grant, G.G.I. Moore and K.F. Swingle, Fed. Proc., Fed. Am. Soc. Exp. Biol., 34 (1975) 759.
2 S.F. Chang, A.M. Miller and R.E. Ober, J. Pharm. Sci., 66 (1977) 1700.
3 C.M. Metzler, G.L. Elfring and A. MacEwen, Biometrics, 30 (1974) 562.
4 J.G. Wagner, Fundamentals of Clinical Pharmacokinetics, Drug Intelligence Publication, Hamilton, IL, 1975.
5 A. Alessandrini, E. Ballarin, A. Bastianon and C. Migliavacca, Clin. Ter., 118 (1986) 177.