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DETERMINATION OF NAFAZATROM IN BODY FLUIDS BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY WITH POST-CHROMATOGRAPHIC DERIVATIZATION

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SUMMARY

A sensitive, accurate and specific high-performance thin-layer chromatographic method has been developed for determination of nafazatrom, a pyrazolinone derivative with antithrombotic and antimetastatic activity. Because of the instability of the drug during clean-up procedures, nafazatrom is recovered from plasma and urine with a single extraction and the extract is spotted directly onto the chromatographic plate. The specificity of the assay was increased by post-chromatographic derivatization with 4-dimethylaminobenzaldehyde. Up to eight primary metabolites with an unchanged position 4 in the heterocyclic ring were also detected and separated chromatographically. The detection limit for nafazatrom is 0.5 ng per spot and 5 ng/ml. The analytical error in the nanogram per millilitre range is less than 10%, low enough for pharmacokinetic studies and for plasma level monitoring in patients.

INTRODUCTION

Nafazatrom (BAY g 6575), 3-methyl-1-[2-(2-naphthyloxy)-ethyl]-2-pyrazolin-5-one, has a pronounced antithrombotic, thrombolytic[1] and antimetastatic[2,3] activity in animal experiments. It has been shown that nafazatrom exerts multiple biochemical effects, such as stimulation of prostacyclin release from the vessel wall of humans and of rats[4–6], stimulation of prostacyclin biosynthesis[7], inhibition of cellular lipoxygenase activity[8] and inhibition of 15-hydroxyprostaglandin dehydrogenase[9].

For pharmacokinetic investigations, an assay method had to be developed suitable for determination of nafazatrom in body fluids in the nanogram per millilitre range. Although nafazatrom is almost completely absorbed, the plasma levels of the unchanged drug are low, suggesting rapid metabolic degradation during first pass through intestinal wall and liver. Furthermore, nafazatrom is unstable at low concentrations in polar solvents and in body fluids, most probably because of sensitivity to oxygen since stabilization could be achieved by addition of cysteine hydrochloride.

On conventional thin-layer chromatographic (TLC) plates, the UV detection limit at 225 nm was 5 ng per spot and 20 ng/ml, which seemed to be sufficient for initial pharmacokinetic studies. Because nafazatrom is also unstable on silica gel, the

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TLC plates had to be pre-developed in a solvent containing acetic acid in order to prevent degradation during the spotting procedure (the extracts from body fluid samples were spotted manually one by one). It was found that *in situ* measurement of the absorbance at 225 nm was not specific enough because of interference with endogenous components that could not be separated chromatographically. On the other hand, a pre-chromatographic clean-up procedure with extractions backwards and forwards, as described for other drugs[10,11], could not be applied because of the chemical instability of nafazatrom. Therefore a post-chromatographic derivatization procedure by reaction of nafazatrom with 4-dimethylaminobenzaldehyde was developed which increased the specificity of the TLC method considerably. In addition, the derivative was absolutely stable on silica gel, hence the TLC plates could be measured at any time after derivatization. In recent years, the TLC procedure for nafazatrom has been improved by applying the high-performance technique[12] and an automatic device for spotting twenty samples simultaneously[13,14].

EXPERIMENTAL

Materials and reagents

All reagents and solvents were of guaranteed reagent grade and were used without further purification. 4-Dimethylaminobenzaldehyde (No. 3059), buffer pH 4.0 (Titrisol No. 9884), L-cysteine hydrochloride (No. 2839) for stabilization of nafazatrom in body fluids and precoated silica gel 60 HPTLC plates 10 × 20 cm without fluorescence indicator (No. 5641) were obtained from Merck (Darmstadt, F.R.G.). For preparation of the derivatization reagent, 2.0 g of 4-dimethylaminobenzaldehyde were dissolved in 100 ml of ethanol (stable for weeks when stored in the refrigerator).

Apparatus

The samples were extracted in 10-ml glass-stoppered centrifuge tubes using a mechanical shaker (Minishaker, Braun, Melsungen, F.R.G.). The extracts were spotted onto the HPTLC plates with an Autospotter (Desaga, Heidelberg, F.R.G.), which was carefully optimized as described elsewhere[13,14]. The HPTLC plates were developed in a twin-trough chamber (No. 25254, Camag, Muttenz, Switzerland). Derivatization was performed using a dipping chamber (No. 124151, Desaga). The plates were scanned at a KM3 chromatogram spectrophotometer (Zeiss, Oberkochen, F.R.G.), attached to a potentiometric recorder (Servogor 210, Metrawatt, Nürnberg, F.R.G.) and coupled to an electronic integrator (Minigrator or SP 4100, both from Spectra-Physics, Santa Clara, CA, U.S.A.). For checking the specificity of the assay procedure for unchanged nafazatrom, the Camag U-Chamber chromatograph (No. 28220) and the anticircular chamber (No. 28650), were applied.

Preparation of nafazatrom standards

Nafazatrom (10.00 mg) was dissolved in 10.00 ml of chloroform (stock solution, 1 μg/μl, stable for 5 days). The stock solution was diluted step by step 1:10 with ethanol to obtain solutions with nafazatrom concentrations of 100, 10, and 1 ng/μl. The ethanolic dilutions were freshly prepared before spiking the blank body fluid samples. To 1.0-ml volumes of blank blood plasma, serum or urine, *ca.* 5 mg of cysteine hydrochloride were added as 15 μl of a freshly prepared aqueous solution of 0.5 g/ml (microlitre pipette).

The samples were spiked with the ethanolic nafazatrom solutions using microlitre syringes with total volumes of 2, 5, 10, and 25 µl. Pooled blood plasma (or serum, where necessary) and pooled urine of healthy volunteers were used for preparation of the nafazatrom standards. Before spiking, each batch of blank body fluid was checked for interfering endogenous components. Batches up to 100 ml each were stored at −30°C. For ethical reasons, human plasma was replaced by bovine plasma whenever possible.

Extraction and spotting

To 1.0 ml of body fluid 1.0 ml of buffer pH 4 was added. Twenty samples, usually eight to ten nafazatrom standards in plasma (or urine) and twelve to ten plasma (or urine) samples with unknown concentrations of nafazatrom, were extracted simultaneously for 10 min, each with 500 µl of chloroform. Aqueous and organic layers were separated by centrifugation for *ca.* 10 min.

The lower chloroform layer was removed using a microlitre syringe (500 µl) and transferred to the sample vessels of the Autospotter. Between two samplings, the microlitre syringe was carefully rinsed with chloroform in order to avoid memory effects. After the transfer of the twenty chloroform extracts, a volume of 40 µl was sucked into the PTFE tubings (twenty extracts simultaneously). The sample vessels were replaced by a HPTLC plate, and spotting was carried out, again twenty samples simultaneously, 1.4 cm from the lower edge of the plate at a rate of 2 µl/min (total spotting time, 20 min). Between two spotting procedures, the PTFE tubings were rinsed simultaneously with chloroform using a solvent trough. The Autospotter was operated under a hood with appropriate ventilation.

Development and derivatization reaction

The HPTLC plates were developed in chloroform–ethanol–acetone (90:5:5) to a height of 5.5 cm (actual separation distance *ca.* 4 cm), air-dried and dipped twice, with an interval of 10 sec, into a 2% solution of 4-dimethylaminobenzaldehyde in ethanol. The organic solvent was removed under a stream of air and the derivatization reaction was completed by heating the plates in an oven for 10 min at 80°C to yield red-orange spots on a white background. The spots are stable for weeks.

Measurements and quantitation

The HPTLC plates were scanned in the reflectance mode at 490 nm (slit width, 0.05 mm; slit length, 3.5 mm; scanning speed, 50 mm/min). The concentrations of nafazatrom in the unknowns were calculated using the calibration line or calibration curve of the plasma (urine) standards of the same plate. In stability tests and in pharmacokinetic studies, each sample extract was usually determined in duplicate on two HPTLC plates, and the result was the mean of these two determinations.

Stability tests

The stability of nafazatrom in body fluids, with and without addition of stabilizing agents, was investigated with homogeneous pools of human plasma and urine and with bovine plasma. Nafazatrom (500 ng/ml) was added as described for the preparation of nafazatrom standards. Samples of 1.0 ml were stored for 24 h at room temperature (air-conditioned laboratory with 21°C), for 3 days in the refrigerator at *ca.* +6°C, and for up to 20 weeks at −30°C.

Quality control

The automatic spotting device was checked at weekly intervals for minimal interchannel differences, in order to fulfill high-performance requirements. The accuracy and precision of the total assay were controlled regularly with recovery experiments, the actual concentrations being unknown to the operators.

RESULTS AND DISCUSSION

Stability in body fluids

At concentrations less than 1 μg/ml, nafazatrom is unstable in polar organic solvents, *e.g.* ethanol and acetone, and in plasma, serum and urine. In human plasma with a nafazatrom concentration of 500 ng/ml, a loss of *ca.* 70% was observed within 8 h at room temperature. Degradation occurs even when the body fluid samples were stored at −30°C. Because the chemical structure of nafazatrom suggests an oxidative attack at C-4 of the pyrazolinone ring, antioxidants were tested as stabilizing agents. Sodium bisulphite (5 mg/ml) and L(+)-ascorbic acid (5 mg/ml) were less efficient than L-cysteine hydrochloride at a concentration of 1 mg/ml, therefore the latter was chosen for stabilization of nafazatrom in body fluids. In pharmacokinetic studies, the blood was immediately centrifuged to separate the blood cells and to transfer the plasma to centrifuge tubes containing cysteine hydrochloride. Urine was collected in bottles with a sufficient amount of cysteine hydrochloride. After the observation that for unknown reasons a cysteine hydrochloride concentration of 2 mg/ml was necessary for stabilization of nafazatrom in dog plasma, the amount added to 1 ml of body fluid was generally increased to 5 mg. The results of stability tests under various conditions are summarized in Table I.

Analytical parameters

The detection limit of the pure substance spotted in chloroform solution on a pre-developed HPTLC plate was 0.2 ng per spot. On HPTLC plates directly from the bench, 0.5 ng per spot was always clearly recognized compared with the background scan, as shown in Fig. 1, with repeated scans of the background and of nafazatrom amounts between 0.5 and 5 ng per spot. Because the yield of the one-

TABLE I

RESULTS OF STABILITY TESTS OF NAFAZATROM IN BLOOD PLASMA AND URINE UNDER VARIOUS CONDITIONS

Body fluid	*Observed time period*	*Number of samples*	*Temperature (°C)*	*Initial conc. of nafazatrom (ng/ml)*	*Conc. of cysteine hydrochloride (mg/ml)*	*Mean recovery (%)*	*Standard deviation*
Human plasma	5 h	5	+21	500	1	99.9	0.8
Human plasma	30 days	6	−30	200	1	101.2	1.7
Bovine plasma	20 weeks	18	−30	500	2	97.0	5.9
Bovine plasma	20 weeks	18	−30	500	5	99.6	6.2
Human urine	6 h	6	+21	1000	1	102.0	2.4
Human urine	18 days	5	−30	1000	1	101.0	6.1

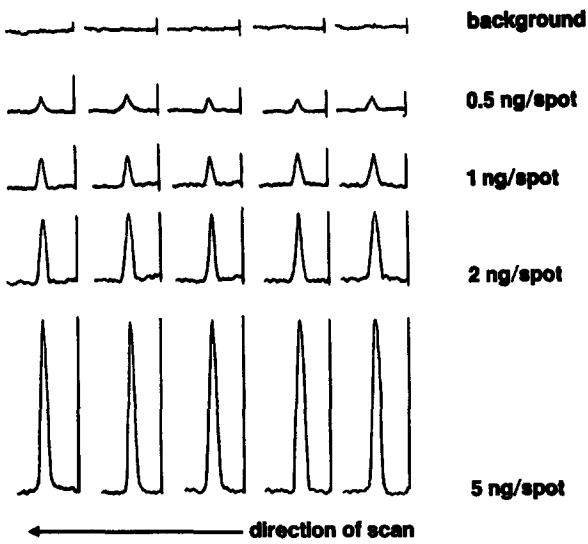


Fig. 1. Repeated scans of nafazatrom spots as pure substance after post-chromatographic derivatization.

step extraction of nafazatrom from plasma and urine is nearly 100% and *ca.* 10% of the extract is spotted on to the plate, the detection limit in body fluids is accordingly 5 ng/ml (Fig. 2). The lower limit of reliable quantitation was 10 ng/ml.

Imprecision was studied for single steps of the assay procedure. The relative standard deviation of 10 repeated scans of one spot in the range 20–200 ng per spot was less than 0.5%. If the automatic spotting device was carefully optimized as described elsewhere[13,14], the imprecision of spotting 20 extracts in volumes of 40 μl each was less than 3%.

The analytical error of the total assay in the concentration range from 10 ng to 10 μg per ml plasma or urine, as judged from controlled blind experiments in the actual concentration range in association with pharmacokinetic studies, is less than 10% (Table II). Additional information about the analytical error can be obtained from the data in Table I.

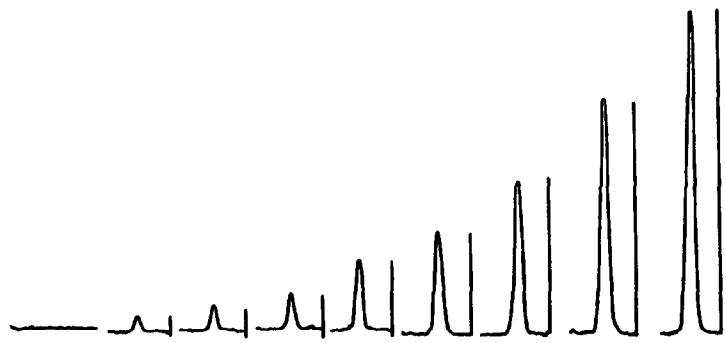


Fig. 2. Peaks of nafazatrom plasma standards in the low nanogram per millilitre range.

TABLE II

ANALYTICAL ERROR OF THE NAFAZATROM DETERMINATION IN PLASMA (RESULTS OF BLIND EXPERIMENTS)

Number of samples	*Range of amount added (ng/ml)*	*Mean percentage found*	*Relative standard deviation*
8	10–110	104.3	9.40
7	84–480	102.1	6.12
9	88–820	96.2	8.92
10	76–618	105.0	6.49
8	140–818	102.9	4.99
6	75–3000	96.2	7.58
8	160–6000	96.8	6.31

Calibration graphs

For each HPTLC plate, a calibration graph was constructed from the peak integrals of the plasma or urine standards. At low concentrations, *e.g.* between 10 and 300 ng/ml, straight or nearly straight calibration lines were obtained (Fig. 3) with the calibration line of the pure substance in the range 1–60 ng per spot. Nafazatrom standards covering a greater range of concentrations, *e.g.* 10–700 ng/ml, as is necessary when analysing samples of patients where absorption and bioavailability is unpredictable, resulted in slightly curved lines (Fig. 4).

Specificity

The HPTLC method for determination of nafazatrom in body fluids can be regarded as "double-specific" because, in addition to the chromatographic process, a derivatization reaction is applied which proved to be specific for a pyrazolin-5-one with two hydrogens in position 4 of the heterocyclic ring. Nafazatrom derivatives

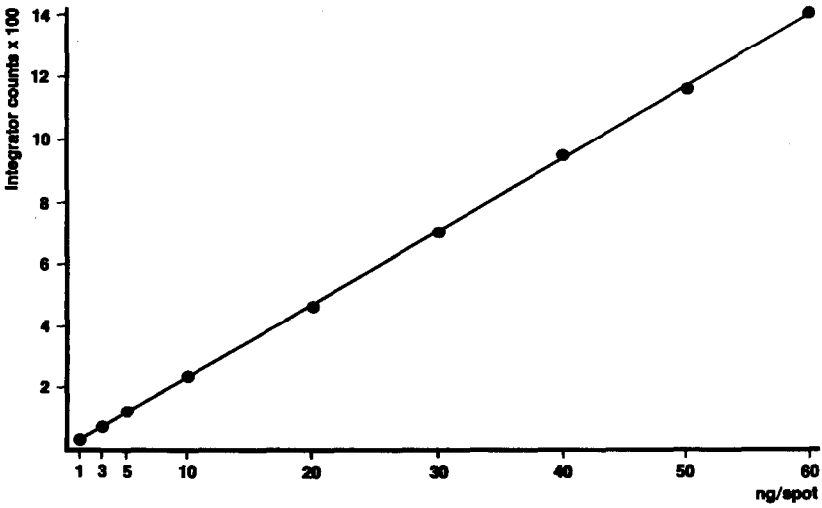
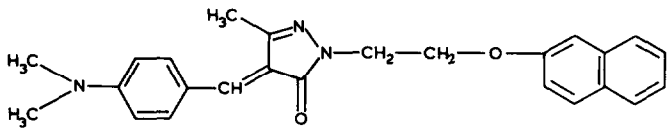


Fig. 3. Calibration line of nafazatrom as pure substance in the low nanogram range on a HPTLC plate.

with one or two alkyl groups in the 4-position of the pyrazolone did not undergo the aldol condensation with 4-dimethylaminobenzaldehyde. Hence the structure is a 4-dimethylaminobenzyliden derivative of nafazatrom, which was also confirmed by mass spectrometry:



Detection of primary metabolites

The derivatization reaction with 4-dimethylaminobenzaldehyde, as already outlined (see Specificity), is specific for pyrazolones with two hydrogens in the 4-position of the heterocyclic ring. Consequently, metabolites that are extractable with chloroform and with structural changes at any other position are detected as well. These metabolites are more polar than nafazatrom and have lower R_F values. In the urine of volunteers after a single oral dose of 20 mg/kg body weight, three primary metabolites were found, designated as M-1, M-2, and M-3. In the urine of a patient under long-term treatment with nafazatrom, a further five metabolites were detected (Fig. 5). At least five of the eight primary metabolites could also be detected in the urine of rats and are, according to their R_F values, identical with those in human urine (Fig. 6). In case one or several metabolites were observed on the HPTLC plate, scanning was carried out from the starting line, *i.e.* metabolites and unchanged nafazatrom are recorded one by one during one scan. In order to be sure that the nafazatrom assay is absolutely specific for the unchanged drug even if primary metabolites are present, the extract from urine was subjected to two-dimensional chromatography and was also checked by circular and anticircular chromatography[15]. The nafazatrom spot always proved to be homogeneous, and there was never any indication that a metabolite with the same or nearly the same R_F value as nafazatrom was present.

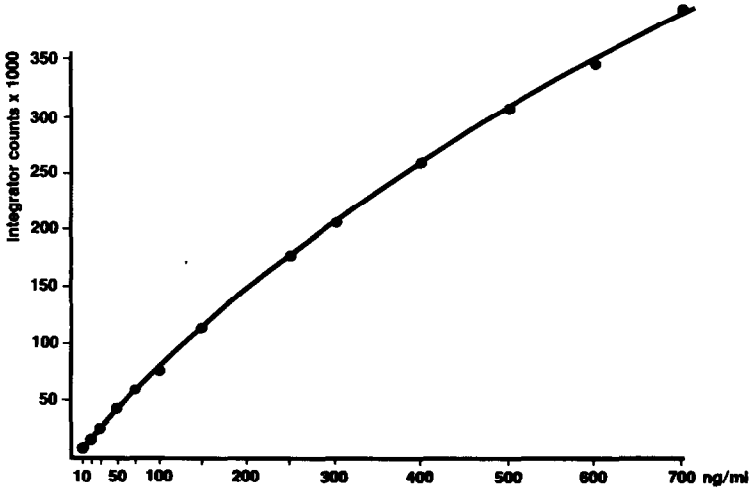


Fig. 4. Calibration line of nafazatrom plasma standards between 10 and 700 ng/ml.

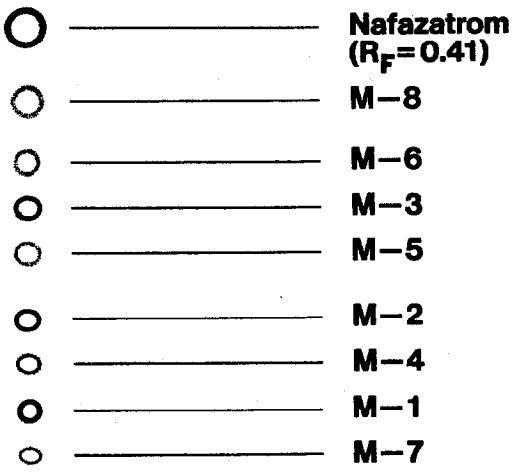


Fig. 5. Chromatographic pattern of eight primary metabolites of nafazatrom (M-1 to M-8) from urine of a patient after oral administration of nafazatrom for several months (HPTLC plate after post-chromatographic derivatization).

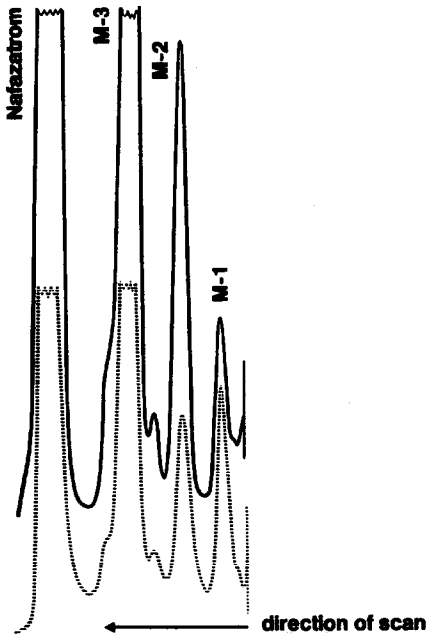


Fig. 6. Scans of the spots of nafazatrom and its primary metabolites in urine of a patient (solid line) and in urine of a rat (broken line).

Possible variations of the assay procedure

For plasma or urine samples with nafazatrom concentrations greater than 1 μg/ml, the assay procedure was slightly modified. Either the sample was extracted with 1 ml of chloroform, or the spotted volume was reduced to 10 or 20 μl per spot, or a smaller volume of body fluid was diluted with buffer pH 7 to give 1.0 ml. In any case, the standards were treated in the same way as the samples with unknown nafazatrom concentrations, hence a control of the actual recovery was never necessary.

Chloroform can be replaced by dichloromethane, if a proper hood is not available and/or the use of chloroform is restricted because of safety regulations. The yield of nafazatrom extraction with dichloromethane is *ca.* 10% lower than with chloroform but this does not influence the results of the determination if the plasma (urine) standards are treated in the same way.

If the sample extracts cannot be spotted simultaneously, the HPTLC plates have to be pre-developed in the solvent system containing *ca.* 5% of acetic acid, to be air-dried and used within 24 h, in order to minimize the degradation of nafazatrom during the spotting procedure.

CONCLUSIONS

The HPTLC method described for determination of nafazatrom in blood plasma and urine is rapid, sensitive, accurate and specific owing to a post-chromatographic derivatization with 4-dimethylaminobenzaldehyde. The HPTLC method has already been successfully applied to pharmacokinetic studies of nafazatrom after oral administration to animals and humans[16].

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