Journal of Chromatography, 487 (1989) 117–124
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4502

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF NESOSTEINE IN HUMAN PLASMA AND URINE

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(First received May 16th, 1988; revised manuscript received September 26th, 1988)

SUMMARY

A high-performance liquid chromatographic method for the quantitation of the mucoactive drug sodium 2-(3-thiazolidinylcarbonyl)benzoate in plasma and urine was developed, involving liquid-liquid extraction under acidic conditions and reversed-phase chromatography using ultraviolet detection at 210 or 235 nm. The extraction efficiency, linearity, limit of detection, precision and accuracy were determined. Products of the main biotransformation of the drug, involving hydrolysis of the amidic bond, do not interfere. The method is selective, precise, reproducible and applicable to studies of the pharmacokinetic behaviour of the drug in humans. Pharmacokinetic parameters derived from six healthy subjects following acute intravenous administration of the drug (150 mg) are presented.

INTRODUCTION

Nesosteine, sodium 2-(3-thiazolidinylcarbonyl)benzoate (Fig. 1), is a mucoactive drug able to normalize the altered tracheobronchial secretions both in animals with experimentally induced bronchitis and in patients with respiratory tract diseases, by improving the rheological characteristics of the tracheobronchial mucus and by increasing the speed of mucociliary transport [1–3].

Preliminary metabolic studies on the urine of rats, dogs and humans showed that the biotransformation of this drug mainly involves hydrolysis of the amidic bond with the formation of phthalic acid and thiazolidine (unpublished results). We have developed a simple, precise, reproducible and selective high-performance liquid chromatographic (HPLC) assay suitable for the quantitation of unchanged nesosteine in human plasma and urine. The method has been applied to pharmacokinetic studies in volunteers.

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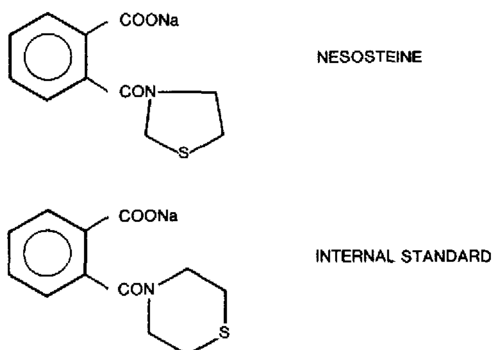

Fig. 1. Structures of nesosteine and of the internal standard.

EXPERIMENTAL

Chemicals and reagents

Nesosteine and the internal standard, sodium 2-(4-thiomorpholinylcarbonyl)benzoate (I.S., Fig 1) were in-house reference standards (purity $\geqslant$ 99%).

Acetonitrile and methanol were of HPLC grade and *n*-hexane, chloroform and orthophosphoric acid of analytical-reagent grade, all from Merck (Darmstadt, F.R.G.), and were used as received.

Standard solutions

Stock solutions of nesosteine (1 mg/ml) and the I.S. (1 mg/ml) were prepared by dissolving the compounds in methanol. Working standards of nesosteine (0.8, 1, 2, 3, 4, 10, 20, 30, 40, 60, 100, 200, 300 and 400 μg/ml in methanol) and of the I.S. (2, 20, 30 and 200 μg/ml in methanol) were prepared from these stock standards. All solutions were stored at $-20°C$ and used within two months of preparation.

Instrumentation and chromatographic conditions

The HPLC apparatus consisted of a Model 5000 solvent metering instrument (Varian, Warrington, U.K.) equipped with a Model 7125 injector (Rheodyne, Berkeley, CA, U.S.A.) fitted with a 10-μl loop and a Varian Model 2050 variable-wavelength UV detector. Reversed-phase HPLC separations were carried out at room temperature ($23 \pm 2°C$) using a guard column (20 mm$\times$4 mm I.D.) packed with Perisorb RP-8 (30–40 μm particle size) (Merck) in series with a LiChrosorb RP-8 (10 μm particle size) (Merck) column (250 mm$\times$4 mm I.D.). For the plasma assay the mobile phase was acetonitrile (solvent A)–25 m*M* phosphate buffer (pH 2.5) (solvent B) (23:77, v/v). For the urine assay the mobile phase consisted of a solvent A–solvent B gradient. The solvent programme was isocratic at 15% A for 2 min, a linear gradient from 15 to 25% A in 10 min and isocratic at 25% B for 4 min. The flow-rate for both assays was 1.0 ml/min. The UV wavelength was set at 210 nm for the plasma assay (nesosteine shows mainly UV absorption) and at 235 nm for the urine assay (to avoid blank interferences).

Sample preparation

Plasma. Aliquots of 0.25 ml of I.S. solution (20 or 2 μg/ml) were dispensed into 10-ml glass centrifuge tubes and evaporated to dryness under a gentle stream of nitrogen. Plasma samples (0.5–1 ml) and 3 ml of *n*-hexane were added to each tube, which were then shaken mechanically for 5 min and centrifuged for 10 min at 600 *g*. The organic layer, containing interfering endogenous substances, was discarded. The aqueous phase, acidified with 0.5 ml of 0.1 *M* sulphuric acid, was extracted twice with 5 ml of chloroform and centrifuged for 10 min at 600 *g*. The combined organic phases were evaporated under dry nitrogen and reconstituted in methanol (0.2–1 ml); 10 μl of this solution were taken for HPLC.

Urine. Aliquots of 0.25 ml of I.S. solution (200 or 30 μg/ml) were dispensed into 10-ml glass centrifuge tubes and evaporated to dryness under a gentle stream of nitrogen. Then 1-ml aliquots of biological samples, obtained by diluting urine with 67 m*M* aqueous phosphate buffer (pH 8) in the range 2–20-fold, and 3 ml of chloroform were added to each tube. The tubes were shaken mechanically for 5 min and centrifuged for 10 min at 600 *g*. The organic layer was discarded. The aqueous phase was acidified with 0.5 ml of 0.1 *M* sulphuric acid and processed as described for plasma.

Calibration and calculation

Calibration graphs were obtained from plasma or urine spiked with standard amounts of nesosteine at concentration ranges expected to include the unknowns. Standards and unknowns were extracted simultaneously as described above. Two plasma calibration graphs were used, one obtained from lower nesosteine concentrations (0.20, 0.25, 0.5, 0.75 and 1 μg/ml) and 0.5 μg/ml I.S. and the other from higher nesosteine concentrations (1, 2.5, 5, 7.5 and 10 μg/ml) and 5 μg/ml I.S. Two urine calibration graphs were prepared using 2.5, 5, 7.5, 10 and 15 μg of nesosteine and 7.5 μg of I.S. and 15, 25, 50, 75 and 100 μg of nesosteine and 50 μg of I.S.

The standard daily responses were collected each day and consecutively cumulated to calculate an overall least-squares linear regression of the peak-area ratios of the drug to internal standard versus drug concentration [4].

Assay validation

The stability of nesosteine in human plasma and urine was investigated. Spiked samples were prepared with drug-free biological fluids and were stored, thawed and analysed together with freshly spiked samples. For each storage period (2 and 4 h at 23 ± 2°C and 1, 2, 3 and 4 weeks at −20°C) and concentration (0.5, 1, 5 and 7.5 μg/ml in plasma; 5, 7.5 and 15 μg in 0.5 ml of urine), three freshly spiked and three stored samples were analysed.

Recoveries of nesosteine and the I.S. from plasma and urine were assessed by comparison of peak areas obtained from the direct injection of the standard stock solutions of the compounds with those found by processing drug-free plasma or urine spiked with nesosteine and I.S. (spiked samples).

The intra-assay precision and accuracy were evaluated by assaying replicate

spiked samples ($n=6$ for each concentration of nesosteine used) of plasma and urine. In addition, the inter-assay precision and accuracy were assessed by analysing each day, for five consecutive days, spiked samples ($n=2$ every day for each concentration) of plasma and urine. Observed concentrations were calculated using the daily cumulative calibration graph. The limit of detection (LOD) was defined as the amount of nesosteine per ml of plasma or of urine giving a signal-to noise ratio of 3 [5].

Pharmacokinetic study

Three male and three female volunteers participated in the preliminary pharmacokinetic study of nesosteine after giving written informed consent.

An intravenous bolus dose of 150 mg of resosteine was administered after an overnight fast and serial plasma samples were obtained over the following 24 h. The total urine output was collected over the intervals 0–2, 2–4, 4–8 and 8–24 h after dosing. All the samples were stored at $-20\,^{\circ}C$ until taken for analysis.

Data for individual plasma concentrations (C) at any time (t) [weighed $1/C^2$] were fitted to be a biexponential equation of the form $C=A\ \exp(-\alpha t)+B\ \exp(-\beta t)$ by using the non-linear least-squares regression program NONLIN [6]. Initial parameter estimates were obtained by means of a laboratory-written peeling program. The elimination half-life ($t_{1/2\beta}$), systemic clearance (Cl_s), steady-state distribution volume (V_{ss}) and renal clearance (Cl_R) were calculated according to an open bicompartmental model, assuming that the elimination occurred from the central compartment [7].

RESULTS AND DISCUSSION

Typical chromatograms obtained from human plasma and urine before and after administration of nesosteine are shown in Fig. 2. Isocratic elution was used for plasma samples whereas gradient elution was required for urine samples, in order to avoid interferences from endogenous compounds. In both these elution systems, phthalic acid, the main acidic biotransformation product of nesosteine, did not interfere.

The stability of nesosteine in human plasma and urine was evaluated at different temperatures, concentrations and storage times. The results are reported in Table I. No significant decrease in concentration ($<10\%$ nominal value) was detected when nesosteine was stored in plasma for 4 h at $25\,^{\circ}C$ and for one month at $-20\,^{\circ}C$ or in urine stored for 4 h at $25\,^{\circ}C$ and for three weeks at $-20\,^{\circ}C$.

Linear calibration graphs were calculated in the ranges 0.2–1 and 1–10 $\mu g/ml$ for plasma and 2.5–15 and 15–100 μg for urine, by consecutively cumulating the data [4] (see the legend to Fig. 2 for examples of calibration graphs). The analysis of variance with an F-test ($\alpha=0.05$) and lack of fit [8] were used to confirm the significance of the regression and the adequacy of the linear model [9].

The LOD for nesosteine in plasma is 70 ng/ml and that in urine is 2.1 $\mu g/ml$. The intra- and inter-assay precision and accuracy for plasma and urine samples at different concentrations of nesosteine are summarized in Table II. The results are satisfactory, showing good reproducibility between experiments. Moreover,

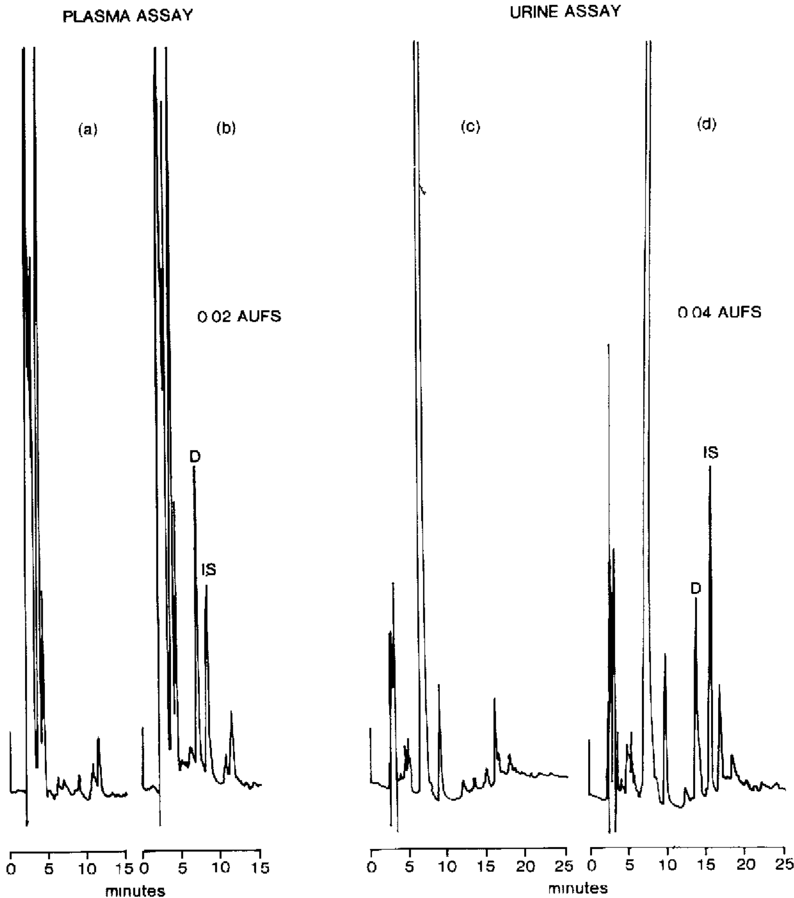

Fig. 2 Representative chromatograms of extracts from (a) drug-free plasma, (b) plasma containing 0 748 μg/ml nesosteine (D) derived from a volunteer who had received 150 mg of the drug intravenously, (c) drug-free urine, (d) urine containing 5.12 μg of D in 0.5 ml derived from the same volunteer as in (b). These concentrations of D were calculated using the day's cumulative calibration graphs $y = -0\ 034 + 1\ 868x$ ($r = 0.994$) and $y = 0.024 + 0.119x$ ($r = 0.996$) for plasma and urine, respectively.

the use of a cumulative procedure allows the precision of the discriminated concentration values to be increased by reducing the confidence interval around them [4].

Plasma and urine recoveries of nesosteine and the I.S. are reported in Table III. Although the nesosteine and I.S. recoveries are not particularly high, they are considered to be acceptable because the extraction procedure described is successful in eliminating most of the potentially interfering endogenous components and because the recovery ratio of the drug to I.S. is highly reproducible.

This methods has been applied to a preliminary pharmacokinetic study following acute intravenous administration of 150 mg of nesosteine to six healthy volunteers. Representative plots of plasma concentration versus time and urinary cumulative excretion of intact nesosteine are depicted in Fig. 3 (volunteer A).

TABLE I

STABILITY OF NESOSTEINE IN HUMAN PLASMA AND URINE ($n=3$)

Nesosteine added (μg)	Storage period[a]	Temperature	Mean nesosteine observed (μg)	Intra-assay C.V.[b] (%)	Deviation from amount added[c] (%)
Plasma (1 ml)					
0.518	4 h	R.T.[d]	0.496	6.4	−4.3
	1 month	−20°C	0.504	7.1	−2.7
1.036	4 h	R.T.	1.011	2.3	−2.4
	1 month	−20°C	1.041	2.8	+0.5
5.18	4 h	R.T.	5.26	1.8	+1.5
	1 month	−20°C	5.12	2.9	−1 2
7.77	4 h	R.T.	7.75	2.5	−0.3
	1 month	−20°C	7.73	2.7	−0 5
Urine (0 5 ml)					
5.18	4 h	R.T.	5.24	2.4	+1.2
	3 weeks	−20°C	5.01	2.6	−3.3
7.77	4 h	R.T.	7.74	1.8	−0.4
	3 weeks	−20°C	7.55	1.9	−2.8
15.54	4 h	R.T.	15.20	1.5	−2.2
	3 weeks	−20°C	14.84	0.6	−4.5

[a]Only the longest storage periods at which degradation is <10% are reported.
[b]Coefficient of variation calculated as $(s/\bar{x})\cdot 100$, where $\bar{x}$ is the mean amount found and s is its standard deviation.
[c]Calculated as $[(\bar{x}-\mu)/\mu]\cdot 100$, where μ is the amount added.
[d]Room temperature (23 ± 2°C).

TABLE II

INTER- AND INTRA-ASSAY PRECISION AND ACCURACY OF THE DETERMINATION OF NESOSTEINE IN HUMAN PLASMA AND URINE

Amount added (μg)	Intra-assay		Inter-assay		Confidence interval ($\alpha=0.05$)[e]	
	C.V.[a] (%)	Accuracy[b] (%)	C.V.[c] (%)	Accuracy[d] (%)	1st day	5th day
Plasma (1 ml)						
0.259	6.4	−0.8	13.1	−2.7	0.398	0 290
0.518	6.3	+7.3	5.9	−4.8	0.359	0 269
0.777	2.7	−4.6	4.7	+0.3	0.376	0.301
Urine (0.5 ml)						
5.180	4.4	−1.1	7.2	+3.0	4.961	4.069
10.360	2.1	+2.8	4.3	−1.2	5.028	3.928
15.540	3.2	+3.4	3.7	+0.8	6.240	4.674

[a]Calculated as $(s/\bar{x})\cdot 100$ where $\bar{x}$ is the mean amount found ($n=6$) and s is its standard deviation.
[b]Calculated as $[(\bar{x}-\mu)/\mu]\cdot 100$, where μ is the amount added.
[c]Calculated as in the first footnote but $\bar{x}$ is the between-days mean amount found ($n=10$) and s is its standard deviation.
[d]See the second and third footnotes.
[e]Mean ($n=2$ every day for each concentration) difference (μg/ml) between upper and lower limits of the Working–Hotelling confidence bands [4].

TABLE III

RECOVERIES OF NEOSTEINE AND INTERNAL STANDARD FROM PLASMA AND URINE ($n=4$)

Nesosteine added (µg)	I.S. added (µg)	Recovery (mean ± S.D.) (%)		Nesosteine/I.S. recovery ratio (mean ± S.D.)
		Nesosteine	I.S.	
Plasma (1 ml)				
0.518	0.507	61.3 ± 1.8	63.5 ± 4.6	0.969 ± 0.057
1.036	0.507	63.1 ± 3.3	66.1 ± 3.9	0.956 ± 0 020
2.590	5.065	64.6 ± 3.8	68.9 ± 3.2	0.938 ± 0.014
5.180	5.065	63.3 ± 1.8	67.4 ± 2.0	0.939 ± 0.020
Urine (0 5 ml)				
7.77	7.598	75.6 ± 2.3	77.7 ± 3.5	0.974 ± 0.020
15.54	50.65	75.7 ± 1.8	76.8 ± 2.6	0.986 ± 0.024

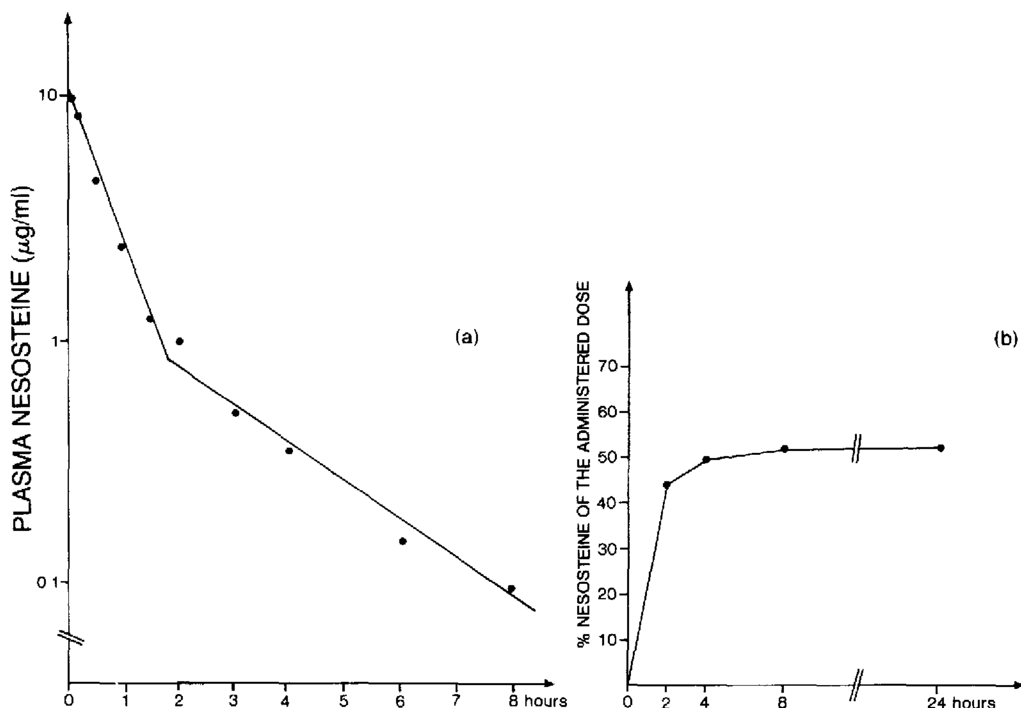

Fig. 3. Plasma (a) concentration-time and (b) cumulative excretion-time profiles of nesosteine following an acute intravenous dose of 150 mg of the drug to the male volunteer A.

Some pharmacokinetic parameters calculated from the plasma curves according to an open bicompartmental model are reported in Table IV.

In conclusion, the proposed HPLC method is sufficiently sensitive and selective for monitoring unchanged nesosteine in pharmacokinetic studies.

TABLE IV

PHARMACOKINETIC PARAMETERS DERIVED FROM SIX VOLUNTEERS FOLLOWING ACUTE INTRAVENOUS ADMINISTRATION OF NESOSTEINE (150 mg)

The parameters are for the free drug: nesosteine does not have any protein binding (unpublished results). The volunteers were 18–26 years old (24.2 ± 3.1) and of 50–62.5 kg body weight (57 8 ± 4.9).

Subject	Sex	$t_{\frac{1}{2}\beta}$ (h)	V_{ss} (l)	Cl_s ($l\,h^{-1}$)	Cl_R[a] ($l\,h^{-1}$)
A	M	1.99	26.1	16 72	8.67
B	F	1.69	18.2	12.04	3.17
C	M	1.69	29.1	17 36	11.99
D	F	1.93	24.6	16.67	5.73
E	F	1.87	28.5	21.40	14.04
F	M	1.83	21.4	15.65	6.70
$\bar{x}$ ± S.D.		1.83 ± 0.12	24.65 ± 4.2	16.64 ± 3.01	8.38 ± 4.05

[a]48.31 ± 17.05% of the administered dose was found in urine as intact nesosteine (0–24 h).

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