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Determination of benzydamine and its N-oxide in biological fluids by high-performance liquid chromatography

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ABSTRACT

A simple, sensitive and selective method for the determination of benzydamine in human plasma and urine, and for benzydamine N-oxide in urine, has been developed using high-performance liquid chromatography in the reversed-phase mode. The limit of reliable determination of benzydamine in plasma was 0.5 ng/ml and that in urine 1 ng/ml; the limit of reliable determination of benzydamine N-oxide in urine was 50 ng/ml. The method has been successfully applied to the analysis of these compounds in biological fluids after administration of intravenous and oral doses of benzydamine to human volunteers.

INTRODUCTION

Benzydamine [1-benzyl-3-(3-dimethylaminopropoxy)-1H-indazole, Fig. 1] is a non-steroidal anti-inflammatory agent with local anaesthetic and analgesic properties [1,2]. It is used both topically and systemically for the treatment of primary or normoreactive types of inflammation; hence its pharmacokinetics has been studied after several routes of administration [1,3,4]. After oral administration to human subjects, benzydamine exhibits almost complete systemic availability; at the time of peak concentrations, it is the major drug-related component circulating in plasma [3]. Metabolites of benzydamine include its N-oxide (detected in both plasma and urine), 1-benzyl-3-hydroxy-1H-indazole and several glucuronic acid conjugates; approximately 70% of an

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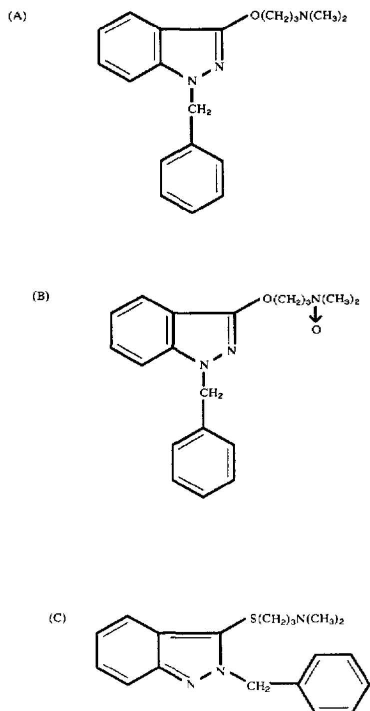

Fig. 1. Structures of (A) benzydamine, (B) benzydamine N-oxide and (C) dazidamine (internal standard).

administered oral dose of benzydamine is excreted in urine as benzydamine and its metabolites [2].

After topical application to the rat, local tissue concentrations of benzydamine are higher than those obtained after oral administration and plasma concentrations are correspondingly lower [1,3,4], thus indicating less systemic exposure after topical than after oral use. Sensitive and specific methods of analysis of benzydamine are required for accurate measurement at these low plasma concentrations.

Published methods for the analysis of benzydamine in biological fluids and topical pharmaceutical preparations involve the use of [^{14}C] benzydamine [5], fluorimetry [4] and high-performance liquid chromatography (HPLC) with either ultraviolet (UV) [6] or fluorescence detection [7]. This paper describes a rapid, sensitive and selective assay for benzydamine and its metabolite, benzydamine N-oxide, using HPLC with fluorescence detection. Unlike previously published methods, this assay uses an internal standardisation procedure with solvent extraction, and may be applied to the analysis of benzydamine in either plasma or urine, and the analysis of benzydamine N-oxide in urine; both com-

pounds are resolved from 1-benzyl-3-hydroxy-1H-indazole, another metabolite. The limits of reliable determination of benzydamine in plasma and urine are 0.5 and 1 ng/ml, respectively, and that of benzydamine N-oxide in urine is 50 ng/ml.

EXPERIMENTAL

Materials

Acetonitrile was HPLC far-UV grade; methanol and diethyl ether were HPLC grade. All other reagents were of analytical grade and all inorganic reagents were prepared in freshly glass-distilled water. Diethyl ether was redistilled prior to use. Standard solutions of benzydamine, benzydamine N-oxide and dazidamine hydrochloride (used as an internal standard) were prepared in methanol and stored in the dark at 4°C under which conditions they were stable for several weeks.

Sample preparation procedure

Benzydamine assay. Samples (1 ml) of plasma or urine were transferred to screw-capped disposable glass extraction tubes, spiked with internal standard and mixed with sodium hydroxide solution (5 *M*, 0.2 ml). The samples were extracted twice by rotary mixing with diethyl ether (5 ml) for 10 min. After centrifugation (2000 *g*, 5 min, ambient temperature), the combined ether extracts were transferred to a glass centrifuge tube and evaporated to dryness under a stream of dry nitrogen at 37°C. The residue was dissolved in mobile phase (0.05 ml) and the total sample injected into the liquid chromatograph.

Benzydamine N-oxide assay. Samples (0.1 ml) of urine were transferred into conical centrifuge tubes, vortex-mixed (10 s) with aliquots of methanol (0.1 ml) containing internal standard, then centrifuged (2000 *g*, 5 min). Aliquots (0.05 ml) of the samples were injected into the liquid chromatograph.

Calibration procedure

Calibration standards of benzydamine in plasma and urine were prepared over the concentration range 0–40 ng base per ml. Samples of control (drug-free) plasma or urine were mixed with appropriate amounts of benzydamine to give samples of concentration 0, 1, 5, 10, 20, 30 and 40 ng base per ml; 200 ng/ml internal standard was added to each sample immediately prior to analysis. For high-level measurements, calibration standards of benzydamine in plasma and urine were prepared over the concentration ranges 0–400 and 0–300 ng base per ml, respectively; 2000 ng/ml (plasma) or 500 ng/ml (urine) internal standard was added to the samples prior to analysis. Calibration standards of benzydamine N-oxide in urine were also prepared over two calibration ranges, the low-level calibration range being 0–1000 ng base per ml and the high-level calibration range 0–40 μg base per ml; internal standard equivalent

to 10 μg/ml (low range calibration) or 700 μg/ml (high range calibration) was added to the samples during analysis. All samples were prepared in bulk and aliquots of appropriate volume (1 ml for the benzydamine assay, 0.1 ml for the benzydamine N-oxide assay) were sub-sampled, stored at −20°C and were stable for up to one month, until required for analysis. One set of calibration standards was taken through the appropriate extraction procedure and analysed with each batch of test samples.

Instrumentation

The chromatograph consisted of an M6000 A pump and WISP™ autosampler (Millipore, Harrow, U.K.) coupled to an LS-4 fluorescence detector (Perkin-Elmer, Beaconsfield, U.K.) operated at an excitation wavelength of 303 nm and an emission wavelength of 377 nm. Chromatograms were recorded using a Trilab II computing integrator (Trivector International, Sandy, U.K.) which automatically calculated peak heights and peak-height ratios.

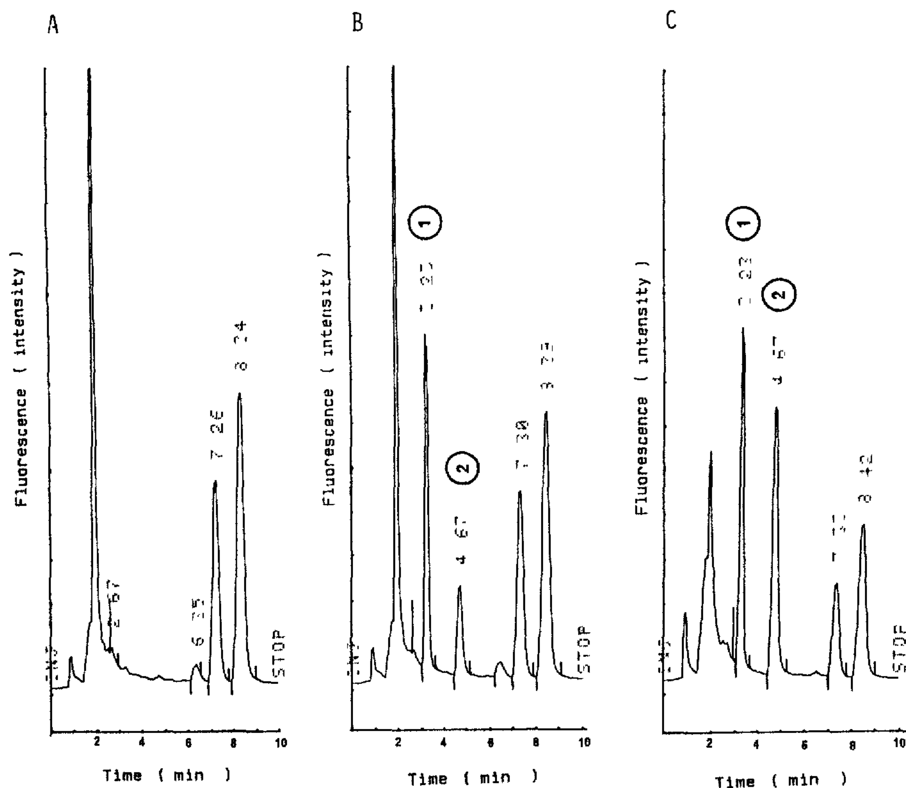

Fig. 2. Chromatograms of plasma extracts (benzydamine assay). (A) Control (blank) plasma; (B) plasma calibration standard containing benzydamine (5 ng/ml); (C) post-dose plasma sample. Peaks: 1 = internal standard; 2 = benzydamine.

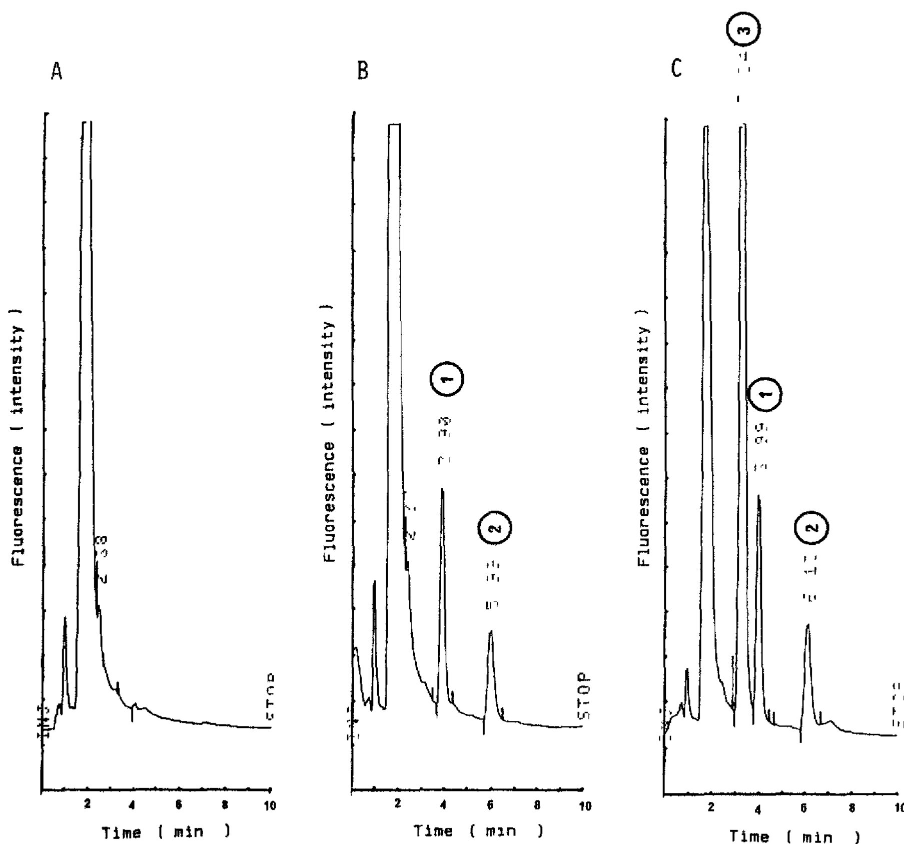

Fig. 3. Chromatograms of urine extracts (benzydamine assay). (A) Control (blank) urine; (B) urine calibration standard containing benzydamine (10 ng/ml); (C) post-dose urine sample. Peaks: 1 = internal standard; 2 = benzydamine; 3 = benzydamine N-oxide.

The column was constructed of stainless steel (30 cm × 0.39 cm I.D.) packed with μBondapak C_{18} (mean particle diameter 10 μm, Millipore). A precolumn (5 cm × 0.2 cm I.D.) constructed of stainless steel and dry-packed with pellicular ODS (particle diameter 37–53 μm, Whatman, Maidstone, U.K.) was installed in front of the analytical column to protect it from contamination.

Chromatography

Chromatography was performed in the reversed-phase mode using a mobile phase of methanol–acetonitrile–water–ammonia solution (25%, v/v), 45:50:5:0.05 (v/v) for the benzydamine assays and 50:40:10:0.05 (v/v) for the benzydamine N-oxide assays. The mobile phase was degassed by vacuum filtration prior to use and passed through the column at a flow-rate of 2 ml/min (adjusted as required). Under the conditions described, benzydamine N-

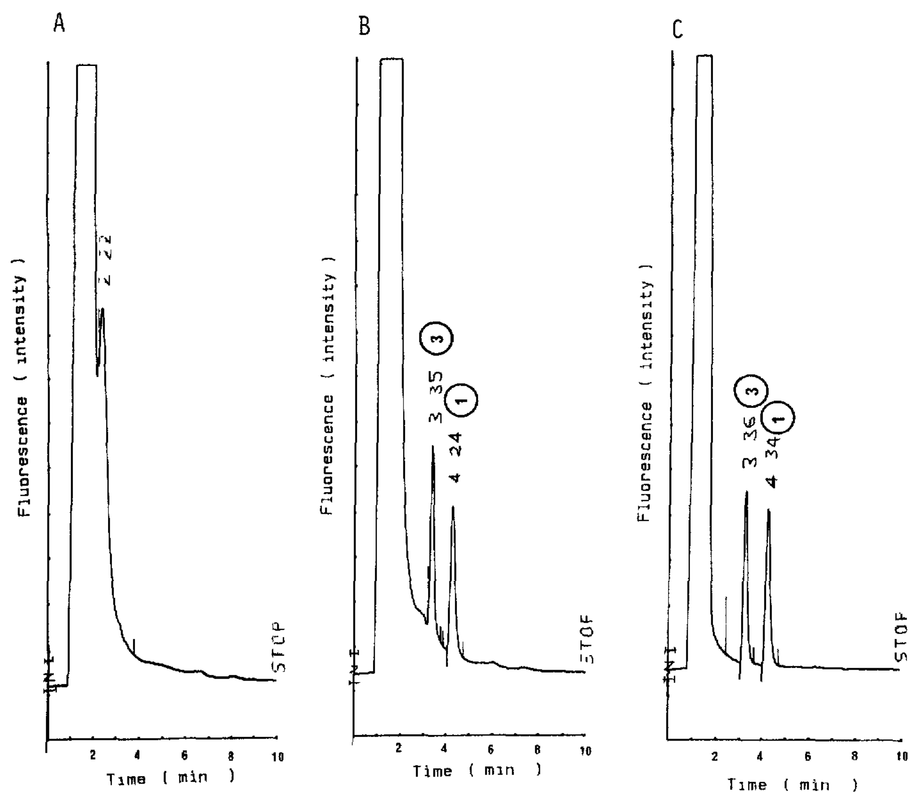

Fig. 4. Chromatograms of urine extracts (benzydamine N-oxide assay). (A) Control (blank) urine; (B) urine calibration standard containing benzydamine N-oxide (300 ng/ml); (C) post-dose urine sample. Peaks: 1 = internal standard; 3 = benzydamine N-oxide.

oxide, internal standard and benzydamine were eluted with retention times of 2.7, 3.2 and 4.6 min, respectively, for the benzydamine assay and 3.3, 4.3 and 6.1 min, respectively, for the benzydamine N-oxide assay (Figs. 2–4); 1-benzyl-3-hydroxy-1H-indazole was eluted with a retention time of 1.2 min under the conditions described for the benzydamine assay.

Studies in human subjects

The method of analysis was applied to plasma and urine samples generated during a study of benzydamine hydrochloride administered as intravenous infusion doses (5 mg) and oral doses (50 mg) to two adult males. Subjects were fasted for 12 h prior to drug administration and for 4 h afterwards; water was available ad libitum throughout this period. Benzydamine hydrochloride was administered in aqueous solution. Blood samples (10 ml) were withdrawn into heparinised tubes prior to dosing and at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 16, 24, 36, 48 and 56 h thereafter; additional samples were taken at 0.08 and

0.17 h after the end of the intravenous infusion. The plasma was separated by centrifugation (2000 *g*, 5 min, ambient temperature) and frozen at $-20°C$. Urine was collected during 12 h before dosing and at 0–2, 2–4, 4–8, 8–16, 16–24, 24–36, 36–48 and 48–56 h thereafter; urine volumes were recorded and the samples frozen. All samples were stored at $-20°C$ until required for analysis, at which time they were thawed, sub-sampled and taken through the extraction procedure previously described. Samples containing benzydamine and benzydamine N-oxide at concentrations in excess of the highest calibration standard were diluted with control plasma or urine prior to reanalysis.

RESULTS AND DISCUSSION

Precision

Extraction and measurement at each of three concentrations over each calibration range was repeated on five occasions. The within-day precision of measurement of benzydamine in plasma, as determined by the coefficients of variation of replicate measurements at concentrations of 1, 20 and 40 ng base per ml, was ±8.9, ±1.4 and ±1.0%, respectively. The precision for benzydamine in urine (replication on four occasions) was ±9.6, ±1.0 and ±0.6% at concentrations of 1, 20 and 40 ng base per ml, respectively, and that of benzydamine N-oxide in urine was ±3.1, ±1.2 and ±2.8% at concentrations of 50, 300 and 1000 ng/ml, respectively (Table I).

Accuracy

Calibration lines for the measurement of benzydamine in plasma and urine, and benzydamine N-oxide in urine, were constructed on each day of test sam-

TABLE I

PRECISION AND ACCURACY OF MEASUREMENT

Sample	Concentration present (ng/ml)	Mean measured concentration (ng/ml)	Number of replicates	Coefficient of variation (%)	Relative error (%)
Benzydamine in plasma	1	1.02	5	8.9	+2.0
	20	19.7	5	1.4	−1.5
	40	39.3	5	1.0	−1.8
Benzydamine in urine	1	1.35	4	9.6	+35.0
	20	19.9	4	1.0	−0.5
	40	40.4	4	0.6	+1.0
Benzydamine N-oxide in urine	50	51.6	4	3.1	+3.2
	300	270.9	5	1.2	−9.7
	1000	925.0	5	2.8	−7.5

TABLE II

CALIBRATION PARAMETERS FOR BENZYDAMINE AND BENZYDAMINE N-OXIDE IN PLASMA AND URINE

Biological fluid	Concentration range (ng/ml)	Slope	Intercept	Correlation coefficient
Benzydamine				
Plasma	0–40	0.05576	0.0053	0.9999
Plasma	0–400	0.00509	−0.0042	0.9999
Urine	0–40	0.04388	−0.0168	0.9997
Urine	0–300	0.01025	−0.0010	0.9999
Benzydamine N-oxide				
Urine	0–40	0.08017	0.0069	0.9995
Urine	0–1000	0.00463	−0.0295	0.9998

ple analysis. In each biological fluid, over each concentration range, plots of peak-height ratio (analyte/internal standard) against concentration were linear ($y=a+bx$, where y is the peak-height ratio and x is the analyte concentration) over the given concentration range ($r=0.999$) (Table II).

The mean of replicate measurements at 1, 20 and 40 ng/ml for the determination of benzydamine in plasma corresponded to 102.0, 98.5 and 98.2% of the true concentrations, respectively. For the determination of benzydamine in urine, replicate measurements at 1, 20 and 40 ng/ml corresponded to 135.0, 99.5 and 101.0% of the true concentrations, respectively, and for that of benzydamine N-oxide in urine, replicate measurements at 50, 300 and 1000 ng/ml corresponded to 103.2, 90.3 and 92.5% of the true concentrations, respectively (Table I).

Recovery

The recoveries of benzydamine and internal standard from plasma and urine were determined by comparison of peak-height ratio measurements of standards taken through the extraction procedures to those of equivalent standards injected into the chromatograph without prior extraction. The mean recovery of benzydamine (20 ng/ml) from plasma and urine was 94% (± 2 S.D., $n=5$) in each case. The mean recovery of internal standard (200 ng/ml) was 92% (± 2 S.D., $n=5$) after extraction from plasma and 103% (± 1 S.D., $n=5$) after extraction from urine.

Specificity and limits of reliable determination

No peaks with retention times similar to those of benzydamine, benzydamine N-oxide or internal standard were present in pre-dose (control) plasma

TABLE III

PLASMA CONCENTRATIONS OF BENZYDAMINE AFTER ADMINISTRATION OF INTRAVENOUS AND ORAL DOSES TO TWO HUMAN VOLUNTEERS

Subject 1 was given a 4.78-mg intravenous and 50-mg oral dose, and subject 2 a 4.48-mg intravenous and 50-mg oral dose

Time (h)	Concentration (ng/ml)			
	Intravenous		Oral	
	1	2	1	2
0[a]	39	72	-	-
0.08	52	60	-	-
0.17	45	67	-	-
0.25	49	65	12	76
0.5	47	55	93	418
0.75	42	55	251	614
1.0	39	58	339	672
1.5	38	53	420	702
2.0	36	47	420	614
3.0	34	34	347	329
4.0	27	34	322	496
6.0	24	32	260	460
8.0	20	26	183	357
12	12	12	132	192
16	9	10	79	122
24	7	6	58	78
36	2	2	17	19
48	1	1	7	7
56	N.D.[b]	N.D.[b]	4	3

[a]End of 8-min infusion.
[b]Not detected.

or urine. A small peak with retention time similar to benzydamine N-oxide was present in post-dose plasma samples but was well resolved from benzydamine. A large peak with a retention time similar to benzydamine N-oxide was present in post-dose urine samples, and it was found necessary to lower the ammonia solution content of the mobile phase to completely resolve this peak for the benzydamine assay.

The limit of reliable determination of benzydamine in plasma and urine was set at 1 ng base per ml, in each case the lowest datum point on the calibration line. In subsequent studies the limit of reliable determination of benzydamine in plasma was set at 0.5 ng base per ml (precision $\pm 17.5\%$, $n=5$). The limit of reliable determination of benzydamine N-oxide in urine was 50 ng/ml. In each biological fluid the signal-to-noise ratio of the analyte, at the limit of detection,

TABLE IV

DERIVED PHARMACOKINETIC PARAMETERS AFTER INTRAVENOUS AND ORAL DOSES TO TWO HUMAN SUBJECTS

Route	Subject No.	Dose (mg)	Half-life (h)	AUC_{α} (ng·h/ml)	Systemic availability	Benzydamine in urine (% of dose)	Benzydamine N-oxide in urine (% of dose)
Intravenous	1	4.78	8.1	484	-	0.4	17
	2	4.48	7.2	555	-	0.5	15
Oral	1	50	8.3	4469	88	0.3	12
	2	50	7.2	6848	111	0.3	15

exceeded 3:1. Representative chromatograms of benzydamine and benzydamine N-oxide in plasma and urine are illustrated in Figs. 2–4.

Studies in human subjects

The method described was successfully applied to the analysis of benzydamine and benzydamine N-oxide in plasma and urine samples generated after administration of single intravenous and oral doses to two healthy male volunteers. Plasma concentrations of benzydamine after intravenous infusion (5 mg) and oral doses (50 mg) of benzydamine hydrochloride are given in Table III and the derived pharmacokinetic parameters in Table IV. Oral doses of benzydamine were rapidly absorbed. Peak plasma concentrations of benzydamine occurred at 1.5–2 h after dosing; thereafter plasma concentrations of benzydamine declined apparently monophasically with a terminal half-life of 7–8 h. The mean systemic availability after oral administration was 100%. Less than 1% of the oral or intravenous doses of benzydamine were excreted unchanged in urine contrary to the results of Köppel and Tenczer [8] who reported that 50–65% of a dose of benzydamine (50 mg) was excreted unchanged in human urine; 12–17% of the dose was excreted as benzydamine N-oxide. Pharmacokinetic data from this study are generally in good agreement with those reported elsewhere [1,3].

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