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Note

Highly sensitive determination of cimetidine and its metabolites in serum and urine by high-performance liquid chromatography

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Cimetidine (Tagamet™, SKF), N-cyano-N-methyl-N'-[2-(5-methyl-1H-imidazoyl-4-yl)methylthioethyl] guanidine, an H_2 receptor antagonist, is used in the treatment of gastrointestinal ulcers and other disease states which may benefit from a reduction in gastric acid secretion. It is oxidized to the sulfoxide (CSO) and hydroxymethyl (COH) metabolites and conjugated to cimetidine-N-glucuronide. In man, after both oral and intravenous [^{14}C]cimetidine, about 80–90% of the dose is excreted in the urine, of which 60–70% is unchanged, 6–10% is CSO, about 4% is COH and virtually all the remainder (24%) is a polar material, probably cimetidine N-glucuronide [1]. Thin-layer chromatography after administration of ^{14}C-labelled cimetidine is the only method reported to date which has been successful in measuring the glucuronide metabolite [1].

In excess of twenty different high-performance liquid chromatographic (HPLC) methods for the measurement of cimetidine have been reported and sensitivities as low as 20 ng/ml have been claimed using reversed-phase chromatography [2,3]. However, to attain this sensitivity for cimetidine precludes the measurement of the more polar CSO and COH which elute with the solvent front or with interfering polar peaks. Five methods are available that simultaneously measure the sulfoxide metabolite [4–8], and only two of these will measure COH [5,7]. The most sensitive of these reported lower limits of detection of 40 ng/ml for cimetidine and CSO and 60 ng/ml for COH [7]. The present paper describes a new HPLC method with excellent sensitivity and selectivity for cimetidine and its CSO and COH metabolites.

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EXPERIMENTAL

Reagents

HPLC-grade methanol and acetonitrile were obtained from Burdick & Jackson Labs. (U.S.A.). Triethylamine (Gold label) was from Aldrich. All other chemicals were Analar grade or better. Cimetidine, its CSO and COH metabolites and the internal standard (I.S.), N:methyl-N′-[3-(imidazol-4-yl)propyl]-cyanoguanidine (the compound has a corporate identification of SK&F 92374), were kindly donated by Smith Kline and French Labs. (Welwyn Garden, U.K.).

Apparatus

Bond Elut™ columns containing 100 mg packing material were from Analytichem International (Harbor City, CA, U.S.A.). The HPLC system was comprised of a Resolve™ C_8 10-μm Radial-Pak cartridge (10 cm × 5 mm) housed in a Radial Compression Module™, a guard column (2.5 cm × 4 mm I.D.) packed with C_{18} Corasil (27–50 μm), a Model 510 pump, a Model 490 multiwavelength-programmable detector [set at 229 nm, and with absorbance units full scale (a.u.f.s.) for serum and urine set at 0.02–0.04 and 0.2, respectively], a WISP™ automatic sample injector (all from Waters, Canada) and a Perkin-Elmer Model 024 flat-bed recorder.

Mobile phase

The mobile phase consisted of 0.01 *M* acetic acid (0.6 g/l) and acetonitrile (84:16, v/v). Both solutions were filtered through a 0.45-μm filter before mixing and degassing. Triethylamine was carefully weighed into a small vial containing approximately 5 ml of the mobile phase, and the contents of the vial were washed into the mobile phase to give a final triethylamine concentration of 0.12–0.16 g/l. The pH of the mobile phase was approximately 4.1.

The mobile phase was pumped through the HPLC system and recycled to the mobile phase reservoir. Adequate mixing of the contents of the reservoir (2 l) was ensured by use of a magnetic stirrer. The mobile phase was discarded after approximately one month of continuous use or when the baseline became too noisy (this occurred only at the sensitive detector attenuation during plasma/serum analyses).

Extraction and chromatography

A modification of the extraction procedure used by Lin et al. [8] was employed. The Vac Elut apparatus which Lin used to speed up the elution of solutions through the columns was not employed. Also we used a chemically similar analogue of cimetidine rather than ornidazole as the internal standard. The Bond Elut C_{18} columns were primed by passing 2.0 ml of each methanol, water and sodium phosphate buffer (0.1 mol/l, pH 7.0) in succession (allowed to drain into a 15-ml centrifuge tube under normal gravity). When about 200 μl of buffer remained on top of the packing up to 500 μl of a sample or standard was added followed by the internal standard (400 ng in 200 μl of buffer for serum, 4 μg in 200 μl of buffer for urine). When the sample had drained to the surface of the packing the column was washed with buffer (two 0.75-ml aliquots) followed by distilled water (0.75 ml). The residual water was removed by gently blowing air through the column using a compressed air line. Methanol (200 μl) was added to the Bond Elut column and after 1 min forced through the column into disposable plastic autosampler tubes using the compressed air line. The elution process was repeated with another 200 μl of methanol.

The methanol eluates (100 μl) were injected onto the HPLC column maintained at room temperature. When more sensitivity was required the methanol eluates were left to evaporate at room temperature in a fume hood for 4–6 h by which time there remained approximately 100 μl of a mixture of methanol–water (this water was the residue remaining on the Bond Elut columns that could not be removed with the compressed air line). After addition of up to 200 μl of mobile phase, 100 μl of the resultant mixture were injected onto the column. Analyses were routinely performed at a flow-rate of 2 ml/min which generated a pressure of about 43 bar on a new column. With regular (weekly) renewal of the guard column packing the C_8 columns lasted many months before separation of the compounds of interest was inadequate.

Standard solutions

Stock solutions of cimetidine, CSO, COH and the I.S. (1 mg/ml) were prepared in distilled water. No instability of the compounds at these concentrations was noted when they were stored at room temperature in the light for up to four months. However, cimetidine at low concentrations in methanol underwent some conversion to the sulfoxide (see Results and discussion).

Standard curves

Standard curves were prepared by spiking known concentrations of cimetidine and the metabolites into serum or urine to obtain twelve calibration samples from 0.01 to 10 μg/ml (serum) and 0.05 to 50 μg/ml (ten-fold dilution of urine). Duplicate aliquots (0.5 ml) of each standard and blank samples were extracted as above.

Data analysis

All compounds were quantitated as peak-height ratio (drug to internal standard) versus concentration. Standard curves were evaluated by least-squares regression analysis.

RESULTS AND DISCUSSION

Chromatography

The novelty of this HPLC method lies in the use of the Resolve C_8 column. The chromatography achieved on the system is shown in Fig. 1. The excellent resolution of the compounds of interest as well as the high sensitivity of the method is illustrated. Good retention of the compounds was achieved so that even the cimetidine sulfoxide was well separated from the solvent front and other endog-

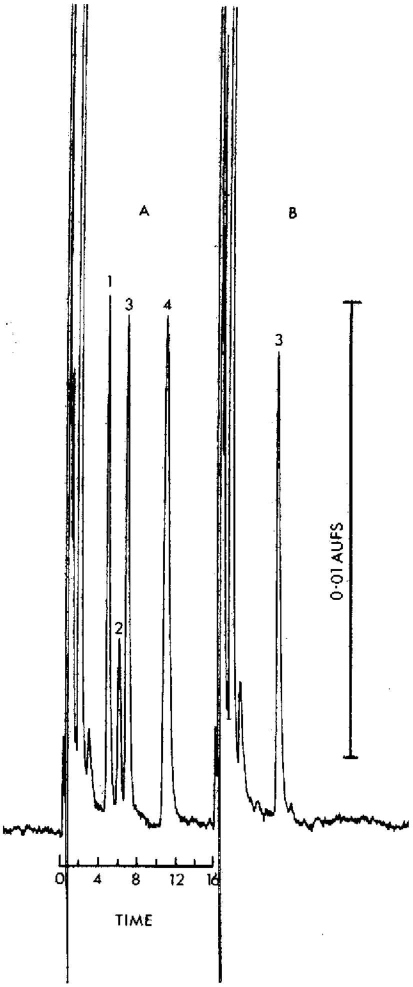

Fig. 1. Chromatograms of (A) extracted human plasma (0.5 ml) that had been spiked with 250 ng cimetidine sulfoxide (1), 125 ng hydroxymethylcimetidine (2), 400 ng I.S. (3) and 500 ng cimetidine (4) and (B) the same extracted plasma spiked only with 400 ng I.S. A 100-μl volume of a total of approximately 300 μl extract was injected onto the column.

enous polar material. Sensitivity was also good, and 3 ng of cimetidine and 2 ng of its metabolites could clearly be measured after injection of these amounts onto the column (3 × background noise).

The peak shape, and to some extent the resolution of the compounds of interest, were influenced markedly by small changes in the concentration of the amine in a manner similar to that described by Lin et al. [8]. Also the optimal amount of the triethylamine varied for different columns.

Endogenous peaks interfered with the measurement of CSO and COH in the serum of some individuals. However, they were more sensitive to changes in the concentration of acetonitrile than the compounds of interest and could often be resolved by small increases (1–4%) in the acetonitrile concentration. Chroma-

TABLE I

PRECISION OF ASSAY

Compound	Recovery ($n=12$) (%)		Serum			Urine		
	Mean	Range	Amount extracted (ng)	Coefficient of variation (%)		Amount extracted (ng)	Coefficient of variation (%)	
				Within-day ($n=8$)	Between-day ($n=8$)		Within-day ($n=6$)	Between-day ($n=6$)
Cimetidine	96	93-101	500	2.7	4.5	5000	1.4	6
Cimetidine sulfoxide	98	94-102	500	2.6	3.1	2500	2.9	6.2
Hydroxymethyl cimetidine	95	92- 98	–	–	–	1250	1.8	1.6

tograms of dilute urine generally had less endogenous material present relative to the peaks of interest.

The method of separation of the compounds is probably a complex mixture of normal- and reversed-phase chromatography. Although this is a reversed-phase system, the silica particles are not end-capped after the bonding process and it is possible that the exposed silanol functions play a major part in the separation process. At the acid pH (4.1) of the mobile phase, cimetidine (pK_a 7.1) and its chemically related analogues are virtually fully ionized. Thus interaction can occur with both the silanol residues and the non polar C_8 bonded phase. The dramatic effect of triethylamine on the retention time of the compounds is probably due to its effect on both the normal- and reversed-phase separation processes. Thus the ionized triethylamine both disturbs cimetidine interaction with the bonded phase by a base pair repulsion effect and with the silanol functions by direct competition.

Extraction

Extraction efficiency was assessed by adding 0.5 or 5 μg of each compound to twelve separate Bond Elut columns together with 500 μl serum or diluted urine. The peak heights of the extracted samples were compared with those of unextracted samples in water. High extraction efficiencies were found (Table I). In addition, within-run coefficients of variation of standards extracted from urine or plasma were excellent (Table I).

The Bond Elut columns have been used repeatedly (up to thirty times) with neither loss of extraction efficiency at the concentrations tested nor increases in the variance of eight standards that were routinely run with each batch of samples. The approximate time required to extract a batch of fourty samples using this method is 90 min. Thus the extraction procedure is cheap, simple, reliable and rapid compared with the solvent extraction method described for the simultaneous determination of cimetidine and its metabolites [5].

Solid-phase extraction has become a popular method in the analysis of cimetidine, and factors that may affect extraction efficiencies are well documented

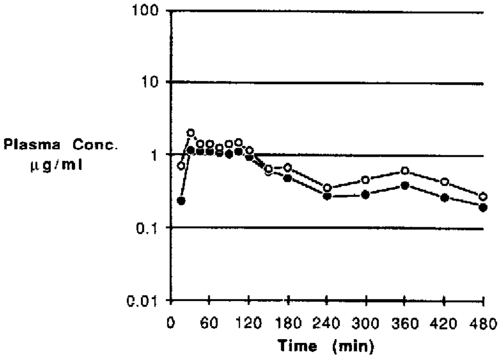

Fig. 2. Plasma concentration-time profile for cimetidine (○) and CSO (●) after an oral dose of cimetidine to a fasted rabbit. The multiple peaks demonstrated here occurred in five out of eleven rabbits studied.

[3,8–11]. The use of the microcolumns in the present paper has advantages over the Sep-Pak system in that the Bond Elut columns are cheaper and require lower fluid volumes for efficient extraction.

Standard curves

The standard curves were linear over the concentrations selected ($r^2 > 0.999$) for all compounds. This linearity was maintained for cimetidine and CSO at low serum concentrations (0.02–1 µg/ml).

When human or rabbit samples containing high concentrations have been analysed we have found it necessary to use smaller volumes of serum (as low as 100 µl) or dilutions of urine (up to 100×) so that the amount of cimetidine undergoing extraction falls within the range of the standard curves. The use of different serum volumes was validated by extracting 100, 200, 500 and 1000 µl of serum

TABLE II

RETENTION TIMES OF DRUGS THAT MAY BE USED CONCURRENTLY WITH CIMETIDINE IN PATIENTS WITH CYSTIC FIBROSIS

A 5-µg amount of each drug was injected onto the column without extraction. N.D. = not detected.

Drug	Retention time (min)	Drug	Retention time (min)
Amikacin	N.D.	Imipenem	2.4
Ampicillin	4	Metronidazole	2
Caffeine	2.4	Sulfamethoxazole	8*
Ceftazidime	2	Theophylline	1.3
Cefoperazone	1.5	Theobromine	1.6
Cephalothin	1.6	β-Hydroxytheophylline	1.5
Ciprofloxacin	N.D.	Ticarcillin	N.D.
Clindamycin	N.D.	Tobramycin	7.2*
Cloxacillin	16*	Trimethoprim	40*

*Possible interference.

($n=4$) containing 250 ng of cimetidine and sulfoxide. No differences in the peak-height ratios or in the coefficient of variation were found.

Stability

It was noted that variable, low levels of the sulfoxide (<5 ng) were produced when methanolic solutions of cimetidine were allowed to evaporate at room temperature or at 37°C. The conversion was not a first-order process since a similar amount of sulfoxide was formed at concentrations from 0.2 to 10 μg/ml. This in vitro instability of cimetidine in methanol had been documented previously by Lin et al. [8] who also noted that further decomposition to an unidentified compound occurred when heat was applied to the sample during a concentration step. Despite these observations no significant differences were found in the concentrations of cimetidine or the sulfoxide when the same sample was analysed immediately or up to 12 h later. However, if the samples were allowed to evaporate completely to dryness some significant increases in the level of CSO were observed which could cause large errors in the estimation of samples containing low concentrations of cimetidine and CSO (i.e. less than 0.2 μg/ml). No instability of cimetidine or its metabolites was found in serum stored at -20°C after reanalysis of samples three months later.

Applications and drug interference

We have used this HPLC method routinely to determine cimetidine and its metabolites from rabbit plasma and urine. These studies attempted to define the cause of the multiple peaks of cimetidine in plasma that are observed after oral cimetidine in man since we had shown that fasted rabbits also demonstrated this effect (Fig. 2). No endogenous peaks were found to interfere with the compounds of interest in this animal species.

We are currently using this HPLC method to study the disposition of cimetidine in patients with cystic fibrosis. Since a number of different drugs are commonly administered to this patient population they were injected onto the column to determine whether interference could occur. The retention times presented in Table II suggested that some commonly used antibiotics have the potential to interfere with the assay. Subsequent extraction of therapeutic concentrations of these antibiotics from serum showed that only the peaks of sulphamethoxazole and trimethoprim interfered with the chromatography.

During the development stages of the analysis, a number of compounds were tried as internal standards. Ranitidine was used initially although it was retained on column for almost twice as long as the cimetidine. Using the same method as described here with a higher flow-rate (3 ml/min) there was adequate separation of CSO and COH from the polar endogenous peaks with ranitidine eluting at about 12 min. This system could easily be developed into a viable method for the estimation of ranitidine using cimetidine or one of its chemical analogues as the internal standard. The extraction efficiency of ranitidine (5 μg) on the Bond Elut system was 85%.

In conclusion, a highly sensitive and selective method for the simultaneous determination of cimetidine and its major metabolites is available with the additional advantages over previous methods of low cost, speed and relative simplicity.

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