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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR AZATADINE IN HUMAN URINE

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SUMMARY

A high-performance liquid chromatographic assay for the quantitative determination of azatadine and a base (1 *M* sodium hydroxide) hydrolyzable conjugate of azatadine in human urine has been developed. Reversed-phase separation of azatadine and the internal standard, 8-chloroazatadine, was accomplished on a 300 × 3.9 mm I.D. μBondapak CN column. Following liquid–liquid extraction from urine, azatadine was quantitatively determined by UV detection at 214 nm. No interferences were observed in the extracts obtained from drug-free urine. Detector response (peak area ratio) was linear from 10 to 2500 ng/ml. This method has been shown to provide accurate and precise determinations of the unchanged and hydrolyzed drug in human urine, following the twice daily oral administration (1–2 mg) of azatadine maleate.

INTRODUCTION

Azatadine maleate, 6,11-dihydro-11-(1-methyl-4-piperidylidine)-5H-benzo-5,6-cyclohepta[1,2-*b*]pyridine dimaleate, is a potent antihistamine[1,2], which is presently marketed alone and in combination with pseudoephedrine sulfate under the trade names Optimine™ and Trinalin™, respectively. It is indicated for the treatment of perennial and seasonal allergic rhinitis as well as chronic urticaria. Standard therapy is 1–2 mg twice daily, the dose being individualized according to each patient's needs and response. Unpublished findings with tritiated drug in man suggested that low-nanogram peak plasma concentrations of unchanged azatadine would be achieved following oral administration of 2 mg of azatadine maleate. There is no published assay methodology which is sensitive enough to quantitate the parent drug in plasma or urine following the administration of therapeutically effective doses. A gas–liquid chromatographic method was reported[3] for the determination of azatadine in the plasma of subjects suspected of drug overdoses; however, the limit of detection was only about 100 ng/ml. The feasibility of an assay designed to determine azatadine in urine following standard therapy was therefore examined. The development and validation of this high-performance liquid chromatographic (HPLC) method is herein described.

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EXPERIMENTAL

Apparatus

Analyses were performed on an HPLC system composed of a WISP (Model 710B) automatic injector (Waters Assoc., Milford, MA, U.S.A.), a Waters Assoc. M6000A pump, and a Waters Assoc. Model 441 UV absorbance detector, equipped with a zinc emission source for monitoring the drug at 214 nm. The detector was connected to a recorder (Model 9176, Varian, Palo Alto, CA, U.S.A.) in order to generate real-time chromatographic tracings. A computer (HP 3357 Lab Automation System, Hewlett-Packard, Palo Alto, CA, U.S.A.) was used to integrate peak areas and automatically calculate results from a single-point-ratio calibration.

Reagents and solvents

Azatadine maleate, 8-chloroazatadine, desmethylazatadine, chlorpheniramine, brompheniramine, pseudoephedrine, and phenylpropanolamine were used as received. All other chemicals, except hexane and acetonitrile (HPLC-quality), were of reagent grade.

Chromatographic conditions

Chromatography was performed on a 300 × 3.9 mm I.D. μBondapak CN (10 μm) column (Waters Assoc.) protected with a silica Guard-Pak™ (Waters Assoc.) pre-column. Reversed-phase separations were accomplished at ambient temperature with a mobile phase consisting of 0.05 *M* monobasic potassium phosphate (pH 5.75)–acetonitrile (2000:900, v/v). The solvent mixture was prepared daily, filtered (0.45 μm), and degassed under reduced pressure before use. A flow-rate of 2.0 ml/min generated a back pressure of *ca.* 150 bar.

Instrument settings

The output voltage (1 V/1 a.u.f.s.) from the Model 441 detector was attenuated with a variable-input adapter to protect the A/D interface with the integrating computer from saturation. The detector sensitivity was adjusted to 0.02 a.u.f.s. for strip-chart recording (10 mV) of each chromatographic analysis.

Extraction procedure for unchanged azatadine

An aliquot (2.0 ml) of human urine was transferred to a 15-ml test tube (16 × 125 mm), fitted with a Polytef-lined screw cap. After the addition of internal standard (1000 ng), prepared in distilled water (0.200 ml), each urine sample was diluted with 1.0 ml of 1 *M* sodium hydroxide and extracted with 6.0 ml of diethyl ether by agitation on an Eberbach (Ann Arbor, MI, U.S.A.) reciprocal shaker for 10 min. Samples were centrifuged for 5 min at 1600 *g* to facilitate separation of the layers. The aqueous portion was frozen in a dry-ice–acetone bath and the organic layer was transferred to a clean 15-ml screw cap test tube. An aliquot (0.5 ml) of 0.05 *M* sulfuric acid was added to the diethyl ether, then shaken and centrifuged as above. The acid portion was frozen as before and the ether was discarded. The drug was re-extracted into 6.0 ml of hexane after alkalinization of the acid layer with 1.0 ml of 1 *M* sodium hydroxide. Following centrifugation, the aqueous layer was frozen in a dry-ice–acetone bath and the hexane was quantitatively transferred to a 20-ml

glass vial. An aliquot (0.100 ml) of concentrated ammonium hydroxide was added to each vial, and the mixture was evaporated to dryness under a stream of nitrogen in a water bath (45°C). The final residue was dissolved in 0.5 ml of the mobile phase and then transferred to an 1.5-ml polypropylene microcentrifuge tube for automatic injection (0.200 ml).

Hydrolysis of azatadine conjugate in urine

An aliquot (2.0 ml) of each urine sample was transferred to a 15-ml test tube (16 × 125 mm), fitted with a Polytef-lined screw cap. After the addition of internal standard (1000 ng), prepared in distilled water (0.200 ml), each specimen was diluted with 1.0 ml of 1 *M* sodium hydroxide and incubated (55°C) in a New Brunswick Scientific (New Brunswick, NJ, U.S.A.) shaker (100 r.p.m.) for 16 h. Samples were allowed to reach room temperature before being processed. The extraction of total drug (unchanged plus hydrolyzed azatadine) proceeded exactly as described above.

Detector response for unchanged azatadine in urine

A standard curve, which reflected an estimated range of unchanged azatadine urinary concentrations achieved following oral (2–4 mg/day) administration of drug, was generated. The linearity of detector response (peak area) was investigated after injections (0.200 ml) of extracts from urine, spiked ($n \geq 5$ per group) to contain 20, 50, 100, 200, and 500 ng/ml of azatadine and a constant concentration (500 ng/ml) of the internal standard. Peak area ratio (azatadine over internal standard) *vs.* azatadine concentration data from the response curve were evaluated by least-squares-fit analysis.

Detector response for azatadine in hydrolyzed urine

A standard curve was generated which reflected an estimated range of azatadine concentrations achieved by hydrolysis of its urinary conjugate following the oral (2–4 mg/day) administration of the drug. Detector linearity was assessed after injections (0.200 ml) of extracts from urine, fortified ($n \geq 5$ per group) with 10, 20, 200, 500, 1000, and 2500 ng/ml of azatadine and a constant concentration (500 ng/ml) of the internal standard. Data from the detector response curve were evaluated by least-squares-fit analysis, as described above for unchanged drug.

Recovery of standards

Urine was collected from several non-fasting human volunteers to generate a drug-free urine pool. The efficiency of drug extraction from urine was determined by the following procedure: Aqueous solutions of azatadine and the internal standard were added to aliquots (2.0 ml) of drug-free urine ($n \geq 5$ per group) to achieve azatadine concentrations of 10, 20, 100, 200, and 1000 ng/ml with a constant internal standard concentration (500 ng/ml). Samples were then extracted as previously described and 0.200 ml of the reconstituted extract was injected for analysis. The recovery of azatadine and the internal standard was calculated by comparing the peak area of both compounds from extracted samples to those obtained from the analysis of equivalent amounts of drug injected directly from stock solutions, diluted with the mobile phase.

Accuracy and precision for unchanged azatadine

Interassay precision and accuracy for the determination of unchanged drug in urine was estimated in the following manner: The analytical results from quality control samples routinely analyzed along with clinical specimens over a 6-month period were grouped by concentration. These included urine samples that were spiked to contain 10, 20, 100, 400, and 1000 ng/ml of azatadine and 500 ng/ml of the internal standard. Daily detector calibration for all analyses were performed on extracts (0.200 ml) from three samples, spiked to contain 100 and 500 ng/ml of azatadine and the internal standard, respectively. Statistics, such as the mean concentration, coefficient of variation (C.V.), and relative accuracy (% bias), were then calculated from the pooled data for each quality control group.

Accuracy and precision for azatadine in hydrolyzed urine

The precision and accuracy for the determination of azatadine in urine following incubation under the conditions of hydrolysis were similarly determined. Analytical determinations from quality control samples routinely analyzed along with clinical specimens over a 6-month period were pooled, and the appropriate statistics were calculated for each concentration group. These included urine samples that were spiked to contain 10, 20, 100, 200, 400, 1000, and 2500 ng/ml of azatadine and 500 ng/ml of the internal standard. Daily detector calibration for these analyses was performed on extracts (0.200 ml) from three samples, spiked to contain 200 and 500 ng/ml of azatadine and the internal standard, respectively.

Selectivity

The chromatographic behavior of desmethylazatadine, pseudoephedrine, phenylpropanolamine, brompheniramine, and chlorpheniramine was evaluated to determine their potential for assay interference. Desmethylazatadine is a minor urinary metabolite of azatadine. Pseudoephedrine is contained with azatadine maleate in the combination product, Trinalin. Phenylpropanolamine, chlorpheniramine, and brompheniramine are drugs commonly found in many "over the counter" cold and allergy remedies. In addition, drug-free human urine was routinely analyzed as described above, and the resultant chromatograms were examined for the presence of UV-absorbing (214 nm) endogenous contaminants which could interfere with the measurement of either azatadine or the internal standard.

Stability of azatadine and conjugate in urine

The stability of azatadine and the internal standard under the conditions of hydrolysis was examined as follows: Replicate ($n = 6$) aliquots (2.0 ml) of drug-free urine were fortified with azatadine and the internal standard at concentrations of 200 and 500 ng/ml, respectively, and then hydrolyzed as above. On the following day, these hydrolysates were extracted along with a group of freshly prepared urine samples ($n = 6$) that were spiked with the same concentration of standards. A direct comparison of the chromatograms, generated from the non-hydrolyzed and base-hydrolyzed extracts, was used to assess the stability of each drug standard. The stability of azatadine in frozen urine was also investigated. Aliquots (2.0 ml) of freshly collected, drug-free urine were spiked ($n = 40$) with azatadine to achieve a concentration of 100 ng/ml and then stored in the frozen state ($-20°C$). At varying time

intervals (0, 10, 24, 38, and 66 days) four samples were removed and analyzed for unchanged azatadine, as described above. The stability of the base-hydrolyzable urinary conjugate was indirectly determined in the following manner. Urine samples from a clinical study in which subjects were treated (*per os*) with 2 mg of azatadine maleate (twice a day) were immediately analyzed for the presence of unchanged azatadine. After storage at −20°C for nearly a year, twelve samples were randomly selected and reanalyzed for the presence of the unchanged drug. A comparison of these results with the initial determinations was used to establish the stability of the conjugate, which undergoes hydrolysis to the parent drug in the presence of 1 *M* sodium hydroxide at elevated temperatures.

RESULTS AND DISCUSSION

Chromatography

Baseline separation was achieved between azatadine (*ca.* 7.5 min) and the internal standard (*ca.* 10.5 min) under the chromatographic conditions described. Optimization of chromatography to accommodate column-to-column variation in performance was accomplished by modest changes in the pH of the mobile phase, altering the ratio of acetonitrile to buffer, or adjusting the flow-rate. Desmethylazatadine, a minor urinary metabolite, had a retention time of 4.5 min and was well separated from azatadine, while both pseudoephedrine and phenylpropanolamine were poorly retained (*ca.* 2.7 min). Chlorpheniramine and brompheniramine had retention times of *ca.* 6.2 and *ca.* 6.6 min, respectively. The presence of these compounds in urine demonstrated little or no potential for interference in this assay. Urine extracts from untreated persons were found to be free of interfering peaks (Fig. 1). Representative chromatograms from urine, spiked with known amounts of azatadine and processed for unchanged and hydrolyzed drug, are shown in Figs. 2 and 3, respectively.

Detector response for unchanged azatadine in urine

A standard curve was generated by using extracts from urine that had been previously spiked to contain known amounts of azatadine (20–500 ng/ml) and internal standard (500 ng/ml). The integrated peak area (μV/s) ratio of azatadine to internal standard was chosen as the quantitative measure of detector response for each azatadine concentration. Weighted (1/variance) regression analysis of these data revealed that the best-fit straight-line relationship between detector response and azatadine concentration had a coefficient of determination (r^2) equal to 0.999. The slope was calculated to be 0.002217/ng, and the y-intercept was determined to be −0.005801.

Hydrolysis of urinary conjugate

During the initial development of this method, aliquots of urine, pooled from several subjects treated with azatadine, were subjected to overnight hydrolysis (16 h) with Glusulase™ (Dupont Pharmaceuticals, Wilmington, DE, U.S.A.) or 1 *M* sodium hydroxide under several different conditions. As can be seen in Table I, maximum hydrolysis with Glusulase at 37°C was achieved at pH 6.5. However, the hydrolytic yield of azatadine was significantly improved by incubation with 1 *M* sodium

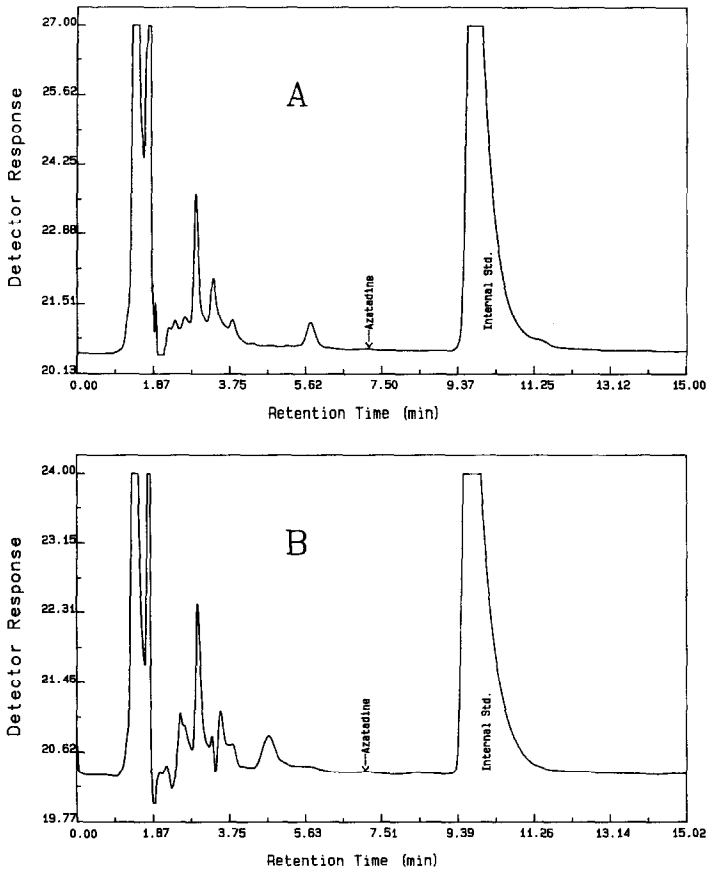

Fig. 1. Chromatogram of an extract from drug-free urine processed for unchanged drug (A) and an extract from drug-free urine following hydrolysis with 1 *M* sodium hydroxide for 16 h at 55°C (B). Both samples were spiked to contain 500 ng/ml of internal standard.

hydroxide at higher temperatures. Although there was a slight increase in the amount of hydrolyzed drug observed at 65°C, the conditions were standardized for hydrolysis at 55°C for the sake of convenience. Additional studies of the time-dependency of sodium hydroxide hydrolysis of the conjugate through 40 h at 55°C, showed that the release of azatadine was essentially complete within 16 h. Chromatograms generated from urinary extracts following base hydrolysis were free of interfering peaks. However, Glusulase treatment contributed a large number of interfering peaks that would have required extensive modification of the extraction procedure and/or chromatographic conditions for low-level (<40 ng/ml) determinations. The conjugate that undergoes hydrolysis in the presence of 1 *M* sodium hydroxide or β-glucuronidase has not been fully characterized. However, cyproheptadine, which is structurally similar to azatadine, is excreted in the urine of man[4] primarily as a quaternary ammonium glucuronide. Efforts directed towards the unequivocal identification of this significant urinary metabolite are continuing in our laboratories.

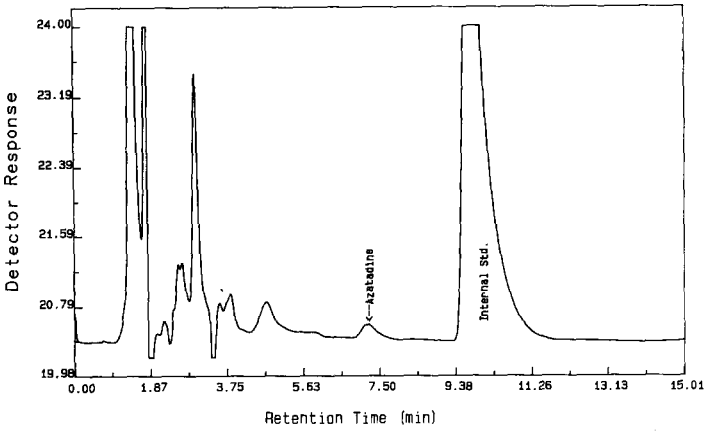

Fig. 2. Representative chromatogram of an extract from urine spiked to contain 10 ng/ml of azatadine. A constant amount (500 ng/ml) of the internal standard was added to the sample before it was processed for unchanged drug.

Detector response for azatadine in hydrolyzed urine

A standard curve was generated using extracts from urine, fortified with azatadine at concentrations ranging from 10 to 2500 ng/ml and then incubated at 55°C for 16 h in the presence of 1 *M* sodium hydroxide. The internal standard concentration remained constant at 500 ng/ml. The integrated peak area (μV/s) ratio of azatadine to internal standard was chosen as the quantitative measure of detector response for each azatadine concentration. Weighted (1/variance) regression analysis on these data revealed that the best-fit straight-line relationship between detector response and azatadine concentration had a coefficient of determination (r^2) equal to 0.997. The slope was calculated to be 0.001897/ng, and the y-intercept was determined to be +0.005691. The y-intercept was not significantly different from zero at the 95% confidence interval.

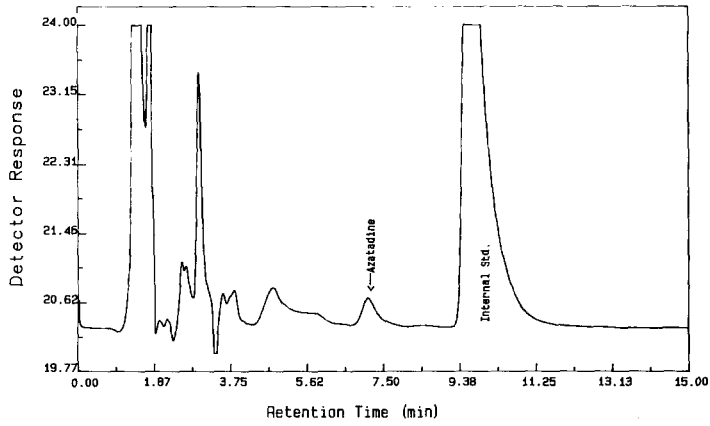

Fig. 3. Representative chromatogram of an extract from urine, spiked to contain 20 ng/ml of azatadine. A constant amount (500 ng/ml) of the internal standard was added to the sample before hydrolysis with 1 *M* sodium hydroxide.

TABLE I

COMPARISON OF THE ENZYMATIC AND BASE HYDROLYSIS (16 h) OF AN AZATADINE CONJUGATE IN HUMAN URINE WITH VARYING pH AND TEMPERATURE

Glusulase hydrolysis at 37°C		*1.0 M Sodium hydroxide hydrolysis*	
pH	*Azatadine released (ng/ml)*	*Temperature (°C)*	*Azatadine released (ng/ml)*
4.5	46.13	22	27.34
5.2	162.77	37	206.83
6.0	324.77	53	669.40
6.5	336.52	65	703.34
7.0	336.30		

Recovery of standards

The average recovery of azatadine from spiked urine samples was found to be 84.63 ± 6.12% S.D. (Table II). The internal standard (500 ng/ml) was extracted with a mean efficiency equal to 85.67 ± 3.99% S.D. Statistical analysis by single-level analysis-of-variance demonstrated that there were no significant differences ($p > 0.05$) among the mean recoveries for azatadine. Therefore, these data, suggest that there is no concentration dependence on extraction efficiency over the range of drug levels (10–1000 ng/ml) in urine that was investigated.

Precision and accuracy for unchanged azatadine

Analytical results for urine spiked with known amounts of azatadine and then processed for the determination of the unchanged drug are shown in Table III. Drug levels in urine, fortified with azatadine at 10, 20, 100, 500, and 1000 ng/ml, were found to range in accuracy (% bias) from −4.2% to +3.8%. The interassay precision (C.V.) of these determinations varied from ±23.8% at the lowest concentration (10 ng/ml) to ±3.72% at 1000 ng/ml.

TABLE II

PERCENT RECOVERY (MEAN ± S.D.) OF AZATADINE AND THE INTERNAL STANDARD FROM HUMAN URINE AT VARYING AZATADINE CONCENTRATIONS

n	*Azatadine concentration (ng/ml)*	*Azatadine recovery (%)*	*Internal standard concentration (ng/ml)*	*Internal standard recovery (%)*
6	10	88.40 ± 10.55	500	86.72 ± 2.50
6	20	83.26 ± 5.07	500	82.59 ± 3.37
5	100	80.54 ± 2.90	500	86.78 ± 3.10
5	200	83.80 ± 1.21	500	82.69 ± 0.71
6	1000	86.33 ± 4.12	500	89.75 ± 4.35
28		84.63 ± 6.12		85.67 ± 3.99

TABLE III

INTERASSAY PRECISION AND ACCURACY FOR THE DETERMINATION OF UNCHANGED AZATADINE IN HUMAN URINE BY HPLC

Theoretical concentration (ng/ml)	*n*	*Mean observed concentration (ng/ml)*	*S.D.*	*C.V. (%)*	*Bias (%)*
10	48	9.97	2.37	23.8	−0.3
20	86	19.16	3.33	17.4	−4.2
100	83	100.9	4.70	4.66	+0.9
400	86	415.1	22.32	5.38	+3.8
1000	13	1036.8	38.60	3.72	+3.7

Precision and accuracy for azatadine in hydrolyzed urine

Analytical data for urine, fortified with known amounts of the drug and processed under the conditions of base hydrolysis, are shown in Table IV. Determinations of the drug in urine, spiked with azatadine at 10, 20, 100, 200, 400, 1000, and 2500 ng/ml, ranged in accuracy (% bias) from −14.6% to +8.5%. The interassay precision (C.V.) for these spiked samples varied from ±10.4% to ±2.82%.

Stability of azatadine and conjugate in urine

Azatadine remained stable for at least two months in urine kept frozen at −20°C. Urine samples, spiked to contain 100 ng/ml of azatadine showed no significant change in concentration over a 66-day period of analysis. The urinary conjugate that is hydrolyzed to the parent drug in the presence of 1 *M* sodium hydroxide at elevated temperatures appeared to remain stable for at least one year under the same storage conditions. This finding was based upon a comparison of the initial determinations of unchanged drug, which were made within days after collection from a clinical study and those made a year later. In addition, no evidence for the hydrolytic decomposition of azatadine or the internal standard was found throughout the 16 h incubation period.

TABLE IV

INTERASSAY PRECISION AND ACCURACY FOR THE DETERMINATION OF AZATADINE FOLLOWING THE HYDROLYSIS OF HUMAN URINE WITH 1 *M* SODIUM HYDROXIDE FOR 16 h AT 55°C

Theoretical concentration (ng/ml)	*n*	*Mean observed concentration (ng/ml)*	*S.D.*	*C.V. (%)*	*Bias (%)*
10	9	8.54	0.89	10.4	−14.6
20	42	18.40	1.27	6.90	− 8.0
100	39	102.2	4.98	4.87	+ 2.2
200	31	202.4	6.95	3.44	+ 1.2
400	8	434.0	12.23	2.82	+ 8.5
1000	16	1025.6	39.42	3.84	+ 2.6
2500	16	2606.9	76.56	2.94	+ 4.3

TABLE V

URINARY EXCRETION OF UNCHANGED AND TOTAL AZATADINE IN A SINGLE SUBJECT TREATED (*PER OS*) EVERY 12 h FOR 3 DAYS WITH 1 mg AZATADINE MALEATE

Time	*Unchanged azatadine (ng/ml)*	*Total* azatadine (ng/ml)*	*Volume (ml)*	*Unchanged azatadine excreted (μg)*	*Total azatadine excreted (μg)*
0–12	9.67	178.60	650	6.29	116.1
12–24	20.85	240.37	670	13.97	161.0
24–36	21.02	287.34	917	19.28	263.5
36–48	16.15	223.76	1132	18.28	253.3
48–60	17.73	355.00	817	14.49	290.0
60–72	14.22	215.56	1375	19.55	296.4
72–84	10.55	200.88	769	8.11	154.5
84–96	10.40	198.71	500	5.20	99.4
			μg (0–96 h) =	105.16	1634.2
			Percent dose** =	3.16	49.1

* Total azatadine = unchanged drug plus azatadine, released from base-hydrolyzed urinary conjugate.

** Total dose = 3.33 mg azatadine free base.

Assay feasibility

Representative urinary excretion data from one of several subjects enrolled in a clinical bioavailability study and treated (*per os*) with 1 mg of azatadine maleate every 12 h for 3 days (total dose = 3.33 mg free base) are shown in Table V. Urine concentration values for the unchanged drug ranged from 9.7 to 21.02 ng/ml, while those for total azatadine were significantly higher and varied from 179 to 355 ng/ml. Conversion of these data to cumulative percent dose revealed that *ca.* 3.2% of the dose was excreted within 96 h as unchanged azatadine, whereas at least 46% was eliminated as a base-hydrolyzable conjugate.

CONCLUSIONS

In summary, a method for the quantitative determination of the unchanged drug and a conjugate of azatadine that undergoes hydrolysis in the presence of 1 *M* sodium hydroxide has been validated for concentrations ranging from 10 to 2500 ng/ml in human urine. Selectivity for azatadine was established in the presence of its desmethyl metabolite as well as the drugs pseudoephedrine, phenylpropanolamine, chlorpheniramine, and brompheniramine, which are commonly administered with azatadine. Levels of the unchanged drug and of a urinary conjugate, which contributes to a major fraction of the total dose, can be determined with equivalent sensitivity by using the same extraction procedure and chromatographic conditions. This assay is currently being employed for the routine measurement of azatadine and its conjugate in urine, following the oral administration of therapeutically effective doses in man.

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