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SIMULTANEOUS DETERMINATION OF ASTEMIZOLE AND ITS DEMETHYLATED METABOLITE IN ANIMAL PLASMA AND TISSUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic procedure has been developed for the determination of astemizole and its primary metabolite in plasma and animal tissues. Both compounds and the internal standard were extracted from alkalinized plasma with heptane—isoamyl alcohol and analyzed using a reversed-phase column and UV monitoring at 254 nm. The detection limits for both compounds were 1 ng/ml of plasma and 5 ng/g of tissue and extraction recoveries were sufficiently high (71—84%). The method was applied to plasma and tissue samples from dogs after repeated oral administration, and to plasma samples from a volunteer taking a 300-mg oral dose of the drug. The results were compared with those obtained by a formerly developed radioimmunoassay.

INTRODUCTION

Astemizole, 1-[(4-fluorophenyl)methyl]-N-{1-[2-(4-methoxyphenyl)ethyl]-4-piperidinyl} -1H-benzimidazol-2-amine (I, Fig. 1), is a new, orally very potent and long-acting histamine H_1 receptor antagonist, devoid of central, sedative and peripheral anticholinergic effects [1]. Astemizole is rapidly absorbed after oral administration, both in man and experimental animals. It undergoes extensive first-pass metabolism and is slowly eliminated, mainly with the faeces [2]. Main metabolic pathways are oxidative O-demethylation, aromatic hydroxylation at the benzimidazole moiety and oxidative N-dealkylation at the piperidine nitrogen, yielding desmethylastemizole (II, Fig. 1) as the major metabolite [2].

Besides radioactivity measurements, drug concentrations in plasma have mostly been measured by a previously described radioimmunoassay (RIA) procedure [3].
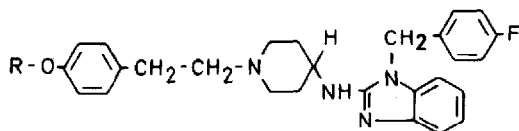
Compound	R
I	CH_3
II	H
III	CH_2-CH_3

Fig. 1. Chemical structure of astemizole (I), desmethylastemizole (II) and the internal standard (III).

The present paper describes a selective high-performance liquid chromatographic (HPLC) method that enables the measurement of both the unaltered drug and its demethylated metabolite in animal plasma and tissues. The method was used to gain further information about the pharmacokinetics of the drug in experimental animals and to allow the validation of the existing RIA procedure.

EXPERIMENTAL

Reagents

Astemizole (R 43 512), desmethylastemizole (R 44 271), 1-[(4-fluoro-phenyl)methyl]-N-{1-[2-(4-hydroxyphenyl)ethyl]-4-piperidinyl}-1H-benzimida-dazol-2-amine and the internal standard (R 44 180), 1-[(4-fluorophenyl)-methyl]-N-{1-[2-(4-ethoxyphenyl)ethyl]-4-piperidinyl}-1H-benzimidazol-2-ami-ne (III, Fig. 1), were obtained from the Life Sciences Products Division of Janssen Chimica (Beerse, Belgium) and were of analytical grade.

Spectrophotometric grade acetonitrile, methanol and n-heptane were used (Uvasol; E. Merck, Darmstadt, F.R.G.). All other reagents were of analytical grade.

Standard solutions

Stock solutions, corresponding to 0.1 mg/ml of methanol, were prepared for compounds I, II and III. Standard solutions were obtained by diluting the stock solution of I and II to concentrations ranging from 0.02 to 20 µg/ml of methanol. To spike the samples with the internal standard, the stock solution of III was further diluted to 1 µg/ml.

Extraction procedure

Plasma. Two millilitres of plasma (unknown samples, drug-free plasma, or plasma standards containing known amounts of the drugs) were transferred to 15-ml glass centrifuge tubes, spiked with 0.1 µg of the internal standard. The solutions were made alkaline with 2 ml of a 0.05 M borax (sodium borate decahydrate) solution, and 4 ml of a heptane—isoamyl alcohol mixture (95:5, v/v) were added. The tubes were carefully rotated for 10 min (10 rpm, Cenco rotary mixer) and then centrifuged (5 min, 1000 g). The upper organic layer

was transferred to a second centrifuge tube by means of a disposable pasteur pipet and the plasma was extracted again with 4 ml of the heptane—isoamyl alcohol mixture. The combined organic layers were back-extracted with 3 ml of 0.05 M sulphuric acid and removed after centrifugation. The remaining acidic phase was made alkaline with concentrated ammonia and extracted twice with 2-ml aliquots of the heptane—isoamyl alcohol mixture. The combined organic layers were finally evaporated to dryness under a gentle stream of nitrogen in a water bath at 55°C.

Tissue samples. Aliquots of the various tissues, previously ground in a Waring commercial blender, were further homogenized (1:4, w/v) in distilled water by means of an Ultra-Turrax TP 18/10 homogenizer. Two millilitres of the resulting homogenates were then extracted as described above.

Calibration procedure

Using the standard solutions of astemizole and its demethylated metabolite, samples of blank control plasma (2 ml) were spiked with both compounds at concentrations ranging from 1 to 100 ng/ml, and with the internal standard at a fixed concentration of 50 ng/ml. These calibration samples were then taken through the extraction procedure described above.

In the same way, standard curves for both astemizole and the metabolite were prepared in blank control animal tissue homogenates (1:4, w/v). Depending on the type of study, the samples were spiked with either high (10 μg/g) or low (0.25 μg/g) concentrations of the internal standard.

Apparatus

The liquid chromatograph used consisted of a Spectra-Physics SP 8700 solvent delivery system, equipped with an SP 8750 organizer module fitted with a 50-μl loop Valco valve injector, and an SP 8300 selectable-wavelength UV—visible detector operating at 254 nm. The separations were achieved using a reversed-phase column (15 cm × 2.1 mm I.D.) packed with 5-μm particle-sized RSiL C18HL (Alltech Europe) by the balanced density procedure by means of an air-driven liquid pump (Haskel). The samples were eluted with acetonitrile—water (50:50) at a constant flow-rate of 0.6 ml/min. To suppress the ionization of the basic functions of the investigated compounds, 0.05% diethylamine was added to the solvent system. Area integrations, peak height measurements, calculations and plotting of the chromatograms were carried out by a Spectra-Physics Model SP 4100 computing integrator.

High-performance liquid chromatography

The various extraction residues were redissolved in 50 μl of methanol by vigorous vortexing, and aliquots as large as possible were injected onto the HPLC column.

Calculations

Ultimate sample concentrations were calculated by determining the peak area ratios of astemizole or desmethylastemizole, related to the internal standard, and comparing these ratios with the standard curves obtained after analysis of the calibration samples.

RESULTS

The recoveries of the extraction procedure for astemizole and desmethyl-astemizole (100 ng) from 2-ml control plasma or tissue homogenate samples are summarized in Table I.

TABLE I

EXTRACTION RECOVERIES FOR ASTEMIZOLE AND DESMETHYLASTEMIZOLE FROM BIOLOGICAL SAMPLES

Compound	Percentage recovery* (mean ± S.D., $n = 5$)	
	Plasma	Tissue
Astemizole (I)	84 ± 3	75 ± 4
Desmethylastemizole (II)	73 ± 4	71 ± 4

*Percentage recovery = recovery of the extraction procedure, obtained after analysis of 100 ng of the appropriate compounds added to 2 ml of control plasma or tissue homogenate.

Retention times under the described chromatographic conditions were 5.8, 1.9 and 8.3 min for compounds I, II, and III, respectively. Fig. 2 shows that no interfering peaks occurred at these retention times and that all compounds eluted as separated symmetrical peaks, although the detection of desmethyl-astemizole (II) was somewhat hindered by the broad solvent front caused by substances originating from the plasma. Linear relationships ($r = 0.999$) were found when the ratio of the peak ara of astemizole and desmethylastemizole to the peak area of their internal standard were plotted on the y-axis against various concentrations of either astemizole or desmethylastemizole on the x-axis. The different correlation coefficients and mathematical expression of the standard curves for I and II in both plasma and tissue calibration samples are summarized in Table II.

The accuracy and precision of the procedure was ascertained by adding different amounts of both compounds to drug-free plasma and analyzing four samples of each concentration with the method described. The results are summarized in Tables III and IV. The detection limits were 1 ng/ml plasma or 5 ng/g tissue for both investigated compounds.

The method described has been used to measure plasma levels of I and II in a male volunteer, taking a 300-mg oral dose, representing 10—30 times the therapeutic dose. The plasma concentration—time profile is depicted in Fig. 3. The method has also been utilized successfully in the analysis of plasma and tissue samples from dogs, after chronic oral treatment with astemizole at a dose level of 10 mg/kg. Some results are given in Fig. 4.
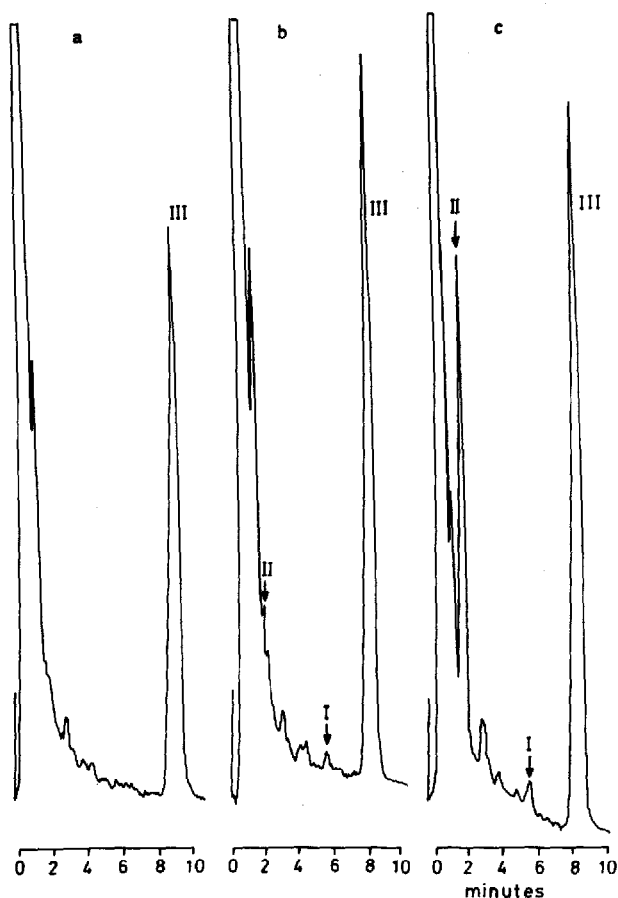
Fig. 2. Chromatograms of extracts from (a) blank control plasma, (b) blank control plasma spiked with 1 ng/ml astemizole and desmethylastemizole, and (c) plasma from a volunteer, 24 h after oral intake of 300 mg of astemizole. I = Astemizole (1.0 and 1.3 ng/ml for chromatograms b and c); II = desmethylastemizole (1.0 and 18.0 ng/ml for chromatograms b and c); III = internal standard (50 ng/ml for all chromatograms). Chromatographic conditions were as indicated in the text.

TABLE II

STANDARD CURVES FOR ASTEMIZOLE (I) AND DESMETHYLASTEMIZOLE (II) IN BIOLOGICAL SAMPLES

Compound	Sample	Internal standard concentration	Range (ng/ml or ng/g)	Regression equation $(Y = aX + b)$[*]		Correlation coefficient	
				a	b	r	n
I	Plasma	50 ng/ml	1—100	0.024	—0.004	0.9999	8
	Tissue	250 ng/g	5—500	0.0047	—0.008	0.9995	10
II	Plasma	50 ng/ml	1—100	0.035	—0.017	0.9999	8
	Tissue	250 ng/g	5—500	0.0069	—0.013	0.9994	10

[*]Y = peak area ratio (astemizole/internal standard, or desmethylastemizole/internal standard); X = astemizole or desmethylastemizole concentration (ng/ml or ng/g).

TABLE III

ACCURACY AND PRECISION OF THE HPLC METHOD FOR THE DETERMINATION OF ASTEMIZOLE IN PLASMA SAMPLES

Theoretical astemizole plasma concentration (ng/ml)	Observed astemizole plasma concentration (ng/ml, mean ± S.D., $n = 4$)	C.V.[*] (%)	Accuracy (%)
1	1.21 ± 0.16	13.1	121.0
2.5	2.56 ± 0.28	11.0	102.4
5	4.85 ± 0.29	5.9	97.0
10	9.30 ± 0.37	4.0	93.0
25	25.0 ± 0.6	2.4	100.0
50	50.6 ± 1.0	1.9	101.2
100	99.8 ± 3.0	3.0	99.8

[*]C.V. = coefficient of variation

TABLE IV

ACCURACY AND PRECISION OF THE HPLC METHOD FOR THE DETERMINATION OF DESMETHYLASTEMIZOLE IN PLASMA SAMPLES

Theoretical desmethyl-astemizole plasma concentration (ng/ml)	Observed desmethyl-astemizole plasma concentration (ng/ml, mean ± S.D., $n = 4$)	C.V.[*] (%)	Accuracy (%)
1	0.90 ± 0.17	18.6	90.0
2.5	2.63 ± 0.38	14.3	105.2
5	4.19 ± 0.20	4.8	83.8
10	10.1 ± 0.5	5.3	101.0
25	25.0 ± 0.5	1.8	100.0
50	50.6 ± 1.0	2.0	101.2
100	99.9 ± 3.3	3.3	99.9

[*]C.V. = coefficient of variation.

DISCUSSION

The extractability of both compounds from plasma was tested in recovery experiments using different alkaline buffer systems with several heptane—iso-amyl alcohol mixtures as the solvent. The maximum extraction recovery was obtained at pH 9 using heptane—isoamyl alcohol (95:5, v/v) and amounted to over 80% and 70% for astemizole and desmethylastemizole, respectively.

In separate experiments, all efforts were made to improve the sensitivity of the method. UV absorbance detection appeared to be more sensitive than fluorescence or electrochemical detection and, although astemizole possesses two absorbance maxima ($\epsilon_0 = 10\ 300$ at 255 nm and $\epsilon_0 = 12\ 300$ at 287 nm), 254 nm was selected because of the better intrinsic sensitivity and the more general accessibility of fixed-wavelength detectors compared to variable-wavelength types.
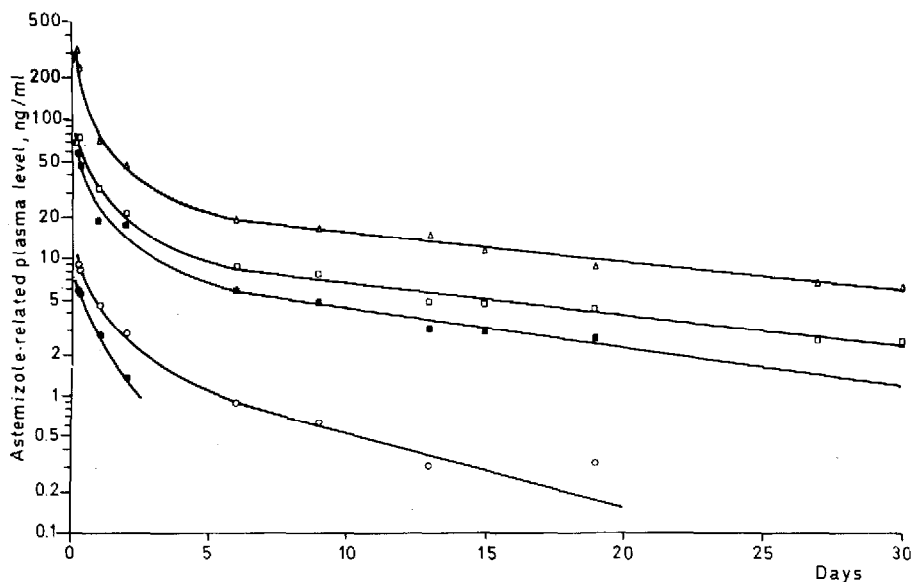
Fig. 3. Astemizole-related plasma levels in a healthy volunteer after ingestion of 300 mg of the drug. (●), Unchanged astemizole (I), as determined by the presented HPLC method; (■), desmethylastemizole (II), as determined by the presented HPLC method; (○), unchanged astemizole, as determined by RIA after the most selective extraction procedure; (□), astemizole and all its phenolic metabolites, as determined by RIA after a single extraction procedure; (△) astemizole and all related metabolites, as determined by direct RIA.
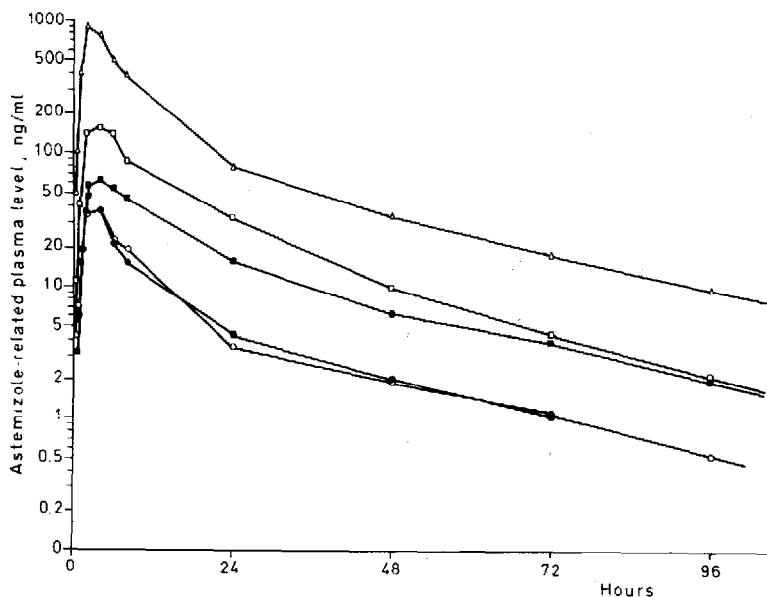

Fig. 4. Mean astemizole-related plasma levels in four dogs, orally treated with the drug at a dose level of 10 mg/kg. (●), Unchanged astemizole (I), as determined by the presented HPLC method; (■), desmethylastemizole (II), as determined by the presented HPLC method; (○), unchanged astemizole, as determined by RIA after the most selective extraction procedure; (□), astemizole and all its phenolic metabolites, as determined by RIA after a single extraction procedure; (△) astemizole and all related metabolites, as determined by direct RIA.

Although a detection limit of 1 ng/ml could be reached, this proved to be insufficient for monitoring therapeutic plasma levels of astemizole, and a more sensitive RIA procedure has been previously developed for this purpose [3]. In order to compare the RIA and HPLC methods for astemizole, samples from experiments in dogs as well as samples from a volunteer taking a 300-mg oral dose, were measured using both assay methods (Figs. 3 and 4). It can be seen that, despite the lack of specificity of the RIA method, there is a striking parallelism between the results obtained for unchanged astemizole (Fig. 3) and for desmethylastemizole (Figs. 3 and 4), as determined by each method. Notwithstanding that very similar results were obtained for the plasma levels of I by both procedures in dogs (Fig. 4), there was a significant overestimation by RIA of the unchanged astemizole plasma levels in man (Fig. 3). This was most probably due to the presence of the N-dealkylated metabolite, 1-[(4-fluoro-phenyl)-methyl]-N-(4-piperidinyl)-1H-benzimidazol-2-amine, which partially interfered in the RIA procedure and which was found to be proportionally more important in man [2]. The latter compound, being a secondary amine with less lipophylic properties, was only extracted to a lower extent, and eluted with the solvent front under the described chromatographic conditions. Hence, the compound as well as all polar metabolites, found by Meuldermans et al. [2], will not interfere in the HPLC procedure presented.

ACKNOWLEDGEMENTS

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