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Short Communication

Detection and determination of free and plasma protein-bound astemizole by thin-layer chromatography: a useful technique for bioavailability studies

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ABSTRACT

A thin-layer chromatographic (TLC) procedure has been developed for the determination of astemizole in plasma as the free and as protein-bound substance. The detection and quantification were performed without using internal standards. In earlier described methods for the estimation of astemizole by high-performance liquid chromatography and radioimmunoassay, only free levels in plasma were quantified, at 3.3% of the total astemizole, with the remaining 96.7% bound to plasma protein and tissue. Our method employs proteolysis of plasma proteins by incubating plasma for 2 h in pepsin. After proteolysis the astemizole is extracted, and a known amount of the extract is spotted on precoated silica gel F 254 plates. Astemizole was quantified using a Shimadzu CS-930 dual-wavelength TLC scanner. The method provides a direct estimate of total astemizole present in the plasma.

INTRODUCTION

Astemizole, 1-[(4-fluorophenyl)methyl]-N-{1-[2-(4-methoxyphenyl)ethyl]-4-piperidinyl}-1*H*-benzimidazol-2-amine, is a histamine H1 receptor antagonist with long duration of action such that oral administration is required only once daily [1]. Pharmacokinetic studies of astemizole have been performed by quantifying the free drug in plasma by high-performance liquid chromatography (HPLC) and radioimmunoassay (RIA) [2]. However, free astemizole in plasma represents only 3.3% of the drug present in the body, the other 96.7% being bound to plasma protein and tissue [1]. Astemizole is rapidly absorbed after oral administration in both humans and animals, and the peak plasma levels were observed at 1–4 h [1,2]. Both RIA and HPLC methods detected the peak plasma levels only when the drug was administered in significantly higher doses (300 mg) than the actual therapeutic dose (10 mg) and when 100 ng of internal standards were used.

This paper describes a reliable and selective thin-layer chromatographic (TLC) method, which enables the determination of total plasma concentrations of aste-

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mizole (both free and protein-bound) when the drug was given orally in doses of 10, 20 and 50 mg to healthy volunteers. The method was also used to obtain further pharmacokinetic information about the drug in experimental animals.

EXPERIMENTAL

Chemicals and reagents

Astemizole was obtained from Auster Pharma (Madrid, Spain). Pepsin (0.35%, pH 1.2, 108 U/mg, P.7125 from Sigma, St. Louis, MO, U.S.A.) was used for proteolysis. Desmethylastemizole is prepared in our R & D Centre. Acetone and benzene (analytical grade) were used for extraction purposes, and *n*-butanol–acetic acid (analytical grade)–double distilled water (12:5:2) was used for developing the TLC plates. Silica gel 60 F 254 precoated plates (Art. 5554, DC-Alufolien, Kieselgel 60 F 254, E. Merck, Darmstadt, Germany) were used for TLC analysis.

Standard solution

Stock solutions of astemizole and desmethylastemizole corresponding to 0.1 mg/ml in methanol were prepared. Standard solutions were obtained by diluting the stock solutions to concentrations ranging from 1 to 20 μg/ml.

Extraction procedure

Plasma. A 2-ml volume of plasma (plasma containing a known amount of the drug, drug-free plasma and unknown plasma) was transferred to a 10-ml glass centrifuge tube.

Incubation. All test samples were incubated for 2 h at 36°C with 2 ml of pepsin (proteolytic enzyme) solution. The incubation time of 2 h was found to be optimum for maximum proteolytic action and complete release of astemizole.

Precipitation. After incubation, the total plasma protein was precipitated by addition of 3 ml of chilled acetone (1°C). The tubes were centrifuged at 666 *g* for 10 min. The supernatant liquid was decanted into a 10-ml test-tube and extracted with two 3-ml volumes of benzene. The combined benzene extract was concentrated and dried completely at 60°C. When plasma protein was precipitated without proteolysis by pepsin, it was observed that astemizole was not completely released by precipitated protein.

Samples of control plasma (2 ml) were spiked with known amounts of astemizole (ranging from 100 to 1000 ng). They were processed through the extraction procedures, and the percentage recovery was calculated.

Thin-layer chromatography

Different residues after extraction were redissolved in 200 μl of methanol by vigorous vortex-mixing, and exactly 100 μl of sample were spotted slowly by using a micropipette. The TLC plates were developed in a glass chamber (25 × 25

× 12 cm) after 2 h saturation with the solvent system *n*-butanol–acetic acid–water (12:5:2). The spots of astemizole were visualized under UV light (280 nm) as quenching of fluorescence, and the total area of each spot was scanned using the TLC scanner. It was observed that desmethylastemizole, the metabolite, moved with solvent front and that astemizole was clearly separated, with an R_F value of 0.45. Determination of astemizole was done by scanning the TLC plates with a Shimadzu dual-wavelength scanner (Model CS-930, Shimadzu, Kyoto, Japan).

Calculations

Sample concentrations were calculated by determining the ratio of the peak area of astemizole in plasma to that of a known concentration of standard, and also by comparison with the standard curve obtained.

RESULTS

The peak area was observed to be dependent on the amount of standard astemizole, and a linear response curve could be drawn (Fig. 1). The recovery of

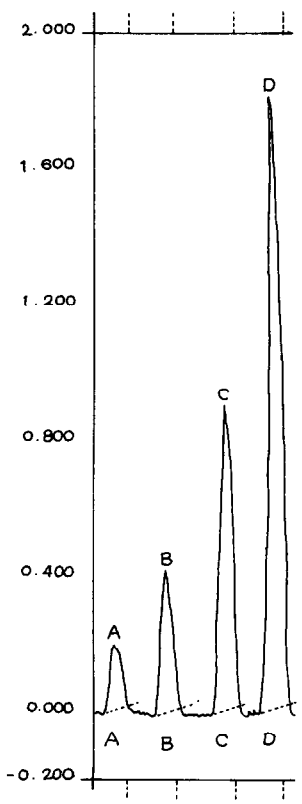

Fig. 1. Linear response of astemizole in plasma (A = 100 ng, B = 200 ng, C = 400 ng, D = 800 ng).

TABLE I

ACCURACY AND PRECISION OF TLC METHOD FOR DETERMINATION OF ASTEMIZOLE IN PLASMA

Concentration added (ng/ml)	Concentration detected (mean ± S.D., n = 5) (ng/ml)	C.V.[a] (%)	Accuracy[b] (%)
100	84.72 ± 10.73	12.37	99.13
200	170.84 ± 14.8	8.70	99.82
400	344.18 ± 25.76	7.48	100.45
800	684.73 ± 14.8	2.07	100.0

[a] Coefficient of variation.

[b] After correction for recovery.

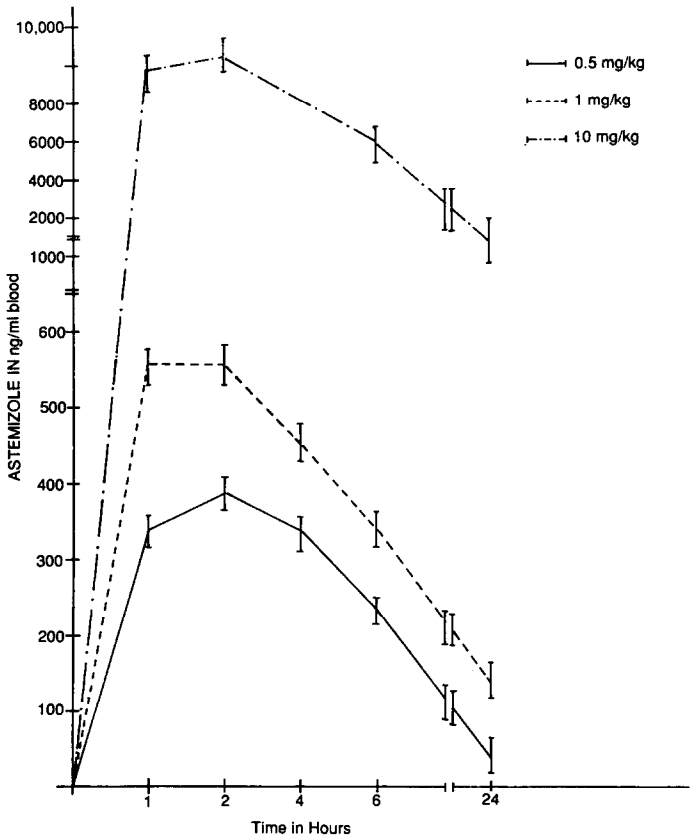

Fig. 2. Plasma levels of astemizole in dogs: the drug was given orally at three different doses.

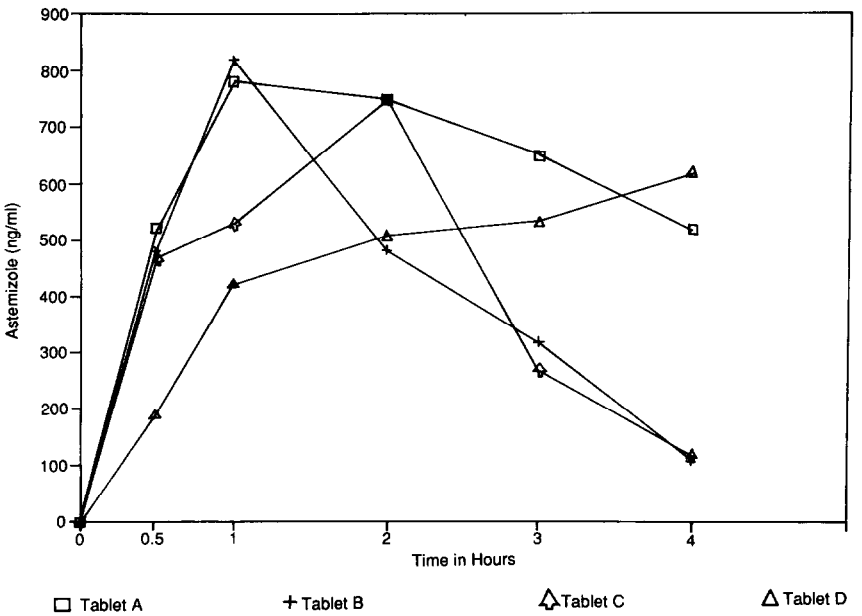

Fig. 3. Comparative bioavailability studies in the same human volunteers with a gap of ten days (as wash out period) of four different marketed tablets of astemizole (dose 50 mg). Plasma levels estimated by the proposed TLC method.

astemizole by the extraction procedure from 2 ml of plasma was found to be 86 ± 2% (n = 5). The R_F value was found to be 0.45–0.5, with λ_{max} = 254 nm.

The solvent system used produced no interfering peaks in the area under the curve, and all the other compounds (desmethylastemizole and other plasma compounds) were distinctly separated with R_F values either at the solvent front or near the starting points. Linear relationships (r = 0.999) were found between peak area of astemizole (plotted on y-axis) and various concentrations (100–1000 ng) of astemizole (plotted on the x-axis). The accuracy, precision and reliability of the procedure were ascertained by adding known concentrations of drug to drug-free plasma and analysing three samples of each concentration by the method described for extraction (Table I).

Plasma astemizole levels were measured in male volunteers, who took 10 mg (the therapeutic dose), 20 mg or 50 mg of the drug. Measurements were taken over 24 h.

The method was also used to measure plasma levels in dogs after oral treatment with astemizole at doses of 0.5 mg, 1 mg and 10 mg/kg orally (Fig. 2).

In order to verify the applicability of this method to different formulations, experiments with four marketed astemizole preparations were conducted at a dose of 50 mg per volunteer (Fig. 3).

DISCUSSION

The proposed TLC method can measure the exact concentrations of plasma astemizole (both free and plasma-bound) at a dose of 50 mg per oral single-dose administration. However, estimation of plasma astemizole at actual therapeutic dose of one 10-mg tablet is possible if 15–20 ml of plasma can be obtained from the patient for each estimation.

A comparison of the proposed method with the earlier HPLC method [2] was carried out by directly using plasma samples of patients who had been given 50 mg of astemizole. Two methods were tried. First, the sample was processed by the HPLC method then spotted on the TLC plates. Second, the sample was processed by the TLC method then injected into the HPLC column and eluted with the reported solvent system. Neither method succeeded in quantifying the drug. This is because of the very small amount of drug extracted in the first method, and the large number of interfering peaks and poor separation observed in the second method. However by using pepsin and processing by the proposed TLC method, it is possible to quantify the true levels of astemizole in plasma (free and protein-bound) by spotting and detecting the samples without using an internal standard and at doses lower than used in HPLC and RIA methods [2].

Astemizole, when given to dogs in concentrations twenty times less than these in the earlier methods, could be estimated and quantified in plasma by the proposed TLC method (Fig. 2).

CONCLUSIONS

The advantages of the proposed TLC method for the estimation of astemizole in plasma can be summarized as follows.

(1) It gives a clear picture of total drug present after absorption (both free and protein-bound astemizole) and thus has direct clinical relevance.

(2) Unlike earlier methods (RIA and HPLC), the TLC method can estimate the drug at appropriate therapeutic doses.

(3) It is economical and faster than earlier methods: on a single plate at least ten to fifteen samples can be analysed in 5–6 h.

(4) Unlike earlier methods, this method does not require an internal standard.

(5) The recovery of the drug is improved (86%).

(6) Estimation of astemizole levels in plasma is possible when the therapeutic dose (10 mg) is given to patients, provided that 15–20 ml of plasma can be collected for each measurement.

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