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SEMI-AUTOMATED GAS CHROMATOGRAPHIC METHOD FOR THE ASSAY OF TIARAMIDE (RHC 2592) IN SERUM SAMPLES

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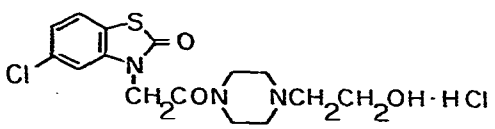
SUMMARY

A rapid and selective semi-automated gas chromatographic method, suitable for the routine assay of tiaramide in serum samples is described. The drug and the internal standard, perphenazine, were extracted with methylene chloride from alkalinized serum samples, and the trimethylsilyl ether derivatives were quantitated by a ^{63}Ni electron-capture detector. Linearity was observed for the range of 0.1—6.0 μg/ml. The average coefficient of variation for all concentration points over a two-week period was 8.5 ± 1.3%. Using an autosampler, the assay rate was 60 to 70 unknown samples in one man-day. The serviceability of the method has been demonstrated in a trial study in which an experimental tablet was given to three dogs. By taking a larger aliquot of the organic extract, a greater sensitivity can be attained if required. This was demonstrated by a trial study with human subjects.

INTRODUCTION

Tiaramide hydrochloride (RHC 2592; Revlon Health Care Group, Tuckahoe, NY, U.S.A.) (I, Fig. 1), 4-[(5-chloro-2-oxo-3(2H)-benzothiazolyl)acetyl]-1-piperazine-ethanol hydrochloride, is marketed in Japan for the treatment of inflammation [1]. Recent studies have shown that RHC 2592 also possesses antianaphylactic and bronchodilatory properties [2, 3]. This agent is now undergoing clinical trial as a new antiasthmatic drug in the U.S.A.



I

Fig. 1. Structure of tiaramide hydrochloride (RHC 2592).

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A gas chromatographic (GC) method for the assay of RHC 2592 in plasma and urine samples has been reported [4]. In this method, RHC 2592 was extracted with ethyl acetate and was then back-extracted with dilute hydrochloric acid. The internal standard was added to the acid extract which was then evaporated to dryness under reduced pressure before derivatization by silylation. The procedure according to this method was lengthy and the evaporation step was time consuming. Therefore, the method was not suitable for the routine assay of a large number of samples.

In order to support formulation development, a rapid and selective semi-automated GC method for the assay of RHC 2592 has been developed in this laboratory such that routine assay of a large number of pre-clinical samples from bioavailability studies in the dog became feasible. The present method permits the assay of 60 to 70 unknown serum samples in one man-day.

EXPERIMENTAL

Gas chromatography

A Perkin-Elmer (Norwalk, CT, U.S.A.) Model Sigma-1 gas chromatograph was equipped with a ^{63}Ni electron-capture detector, and a data station for peak integration and chromatogram display. A 1.8 m × 0.2 cm I.D. glass column packed with 3% OV-17 on 80—100 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.) was used.

The gas chromatograph was operated in the isothermal mode at 300°C, the injector at 315°C and the detector at 350°C. Nitrogen was used as the carrier gas and the detector purge gas, the respective flow-rates of which were 25 and 35 ml/min.

Reagents and standards

Acetonitrile, methylene chloride and ammonium hydroxide (Fisher Scientific, Fair Lawn, NJ, U.S.A.) were used as provided. Perphenazine (II), 4-[3-(2-chlorophenothiazin-10-yl, propyl]-1-piperazine-ethanol (gift from Schering, Kenilworth, NJ, U.S.A.) was selected as the internal standard, and Tri-Sil/BSA, Formulation P (Pierce, Rockford, IL, U.S.A.) was used as the derivatizing reagent. Control dog serum (Pel-Freez Biologicals, Rogers, AR, U.S.A.) was used for the preparation of standard curves. Standard solutions of RHC 2592 (10.00 mg%) and perphenazine (10.00 mg%) were prepared by dissolving the pure drug in distilled water and were stored at 5°C.

Procedure

A series of standard serum samples with respective concentrations of 6.0, 4.0, 2.0, 1.0, 0.4, 0.2 and 0.1 μg/ml was prepared by adding different aliquots of tiaramide hydrochloride standard solution into a series of PTFE-lined screw-capped tubes containing 1.0 ml of control dog serum. An aliquot of perphenazine standard solution (200 μl) was also added to each tube as an internal standard. These standard serum samples were used to generate a standard curve from which the unknown serum sample concentrations were calculated. Similarly, the same amount of the internal standard was added to serum samples of unknown tiaramide hydrochloride concentration.

To both the standard and unknown serum samples, 1 ml of water and 1 ml of 0.5 *N* ammonium hydroxide solution were added, and the samples were mixed gently. The extraction of the drug and the internal standard was carried out with an aliquot of 5.0 ml methylene chloride by shaking the tubes in a reciprocating shaker for 20 min. After phase separation by centrifugation, an aliquot of 0.5 ml of the organic extract was transferred to an autosampler vial. The extract was then evaporated to dryness at 35—40°C with a stream of nitrogen.

The dried extract was re-dissolved in 525 μl of acetonitrile and 75 μl of the derivatizing reagent. After the vials were sealed with a crimper and the contents were well-mixed, the vials were placed in a block heater at 75°C for 1 h to ensure complete reaction.

Sample injections were made by an auto-sampler (Perkin-Elmer). Sampling parameters specified an injection volume of 2 μl and a sampling time of 12 min. The 12-min time interval between injections allowed for proper detector re-equilibration.

RESULTS AND DISCUSSION

Under the present chromatographic conditions, the silylated derivatives of RHC 2592 and that of the internal standard (perphenazine) were eluted at 2.9 and 4.1 min, respectively. Fig. 2A is a typical chromatogram of a serum standard sample containing 2.0 μg/ml of RHC 2592 and 20.0 μg/ml of perphenazine. Fig. 2B shows the chromatogram of a serum sample from a dog

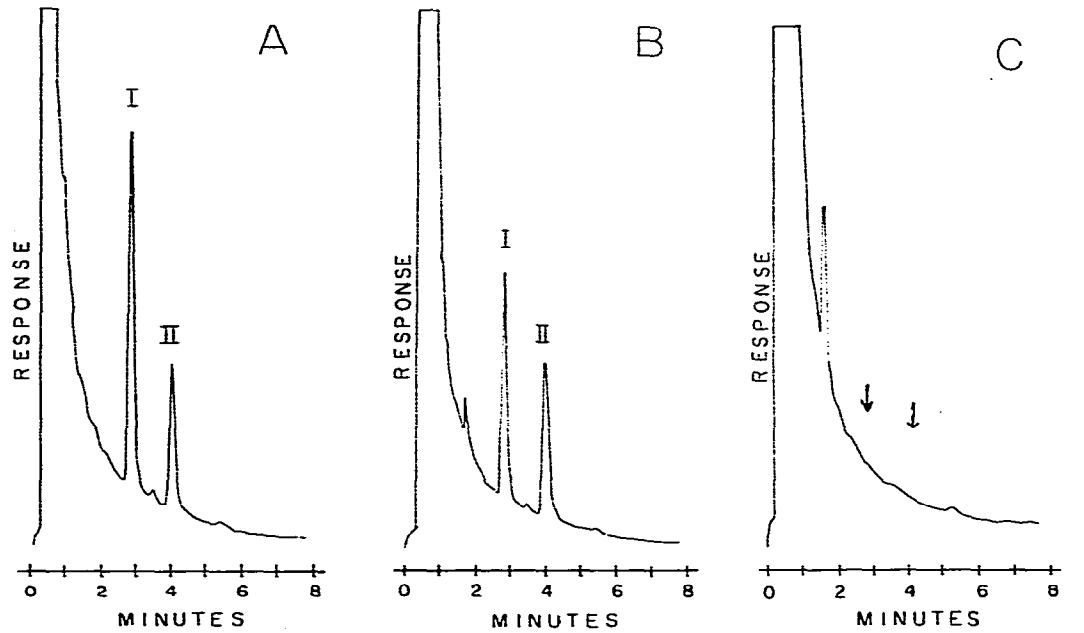

Fig. 2. Chromatograms of serum samples assayed as described. (A) Control dog serum sample which was spiked with 2.0 μg/ml of RHC 2592 (I) and 20.0 μg/ml of perphenazine (II) (internal standard); (B) serum sample from a dog 4.0 h after an oral dose of I, spiked with II; (C) pre-dose dog serum sample showing no interfering peaks at the retention times of I and II (indicated by arrows).

4.0 h post-dose. The peak at 2.9 min represents 1.1 μg/ml of RHC 2592. Fig. 2C is a typical chromatogram of a pre-dose serum sample from a dog. No interfering peaks were observed at the retention time of the derivatives of either RHC 2592 or perphenazine.

Gas chromatography—mass spectrometry

The silylated derivative of RHC 2592 from the extract of a serum standard sample, under the present reaction conditions, was investigated by GC—mass spectrometry (MS) using electron-impact ionization (Finnigan 4000 gas chromatograph—mass spectrometer, Finnigan, Sunnyvale, CA, U.S.A.). The GC—MS data show a molecular ion at *m/z* 427, a base ion at *m/z* 324, and fragmentation ions at *m/z* 412 and 198. The fragmentation ion at *m/z* 412 and the base ion at *m/z* 324 correspond to the loss of a $-CH_3$ and a $-CH_2-O-Si-(CH_3)_2$ group, respectively. The subsequent loss of a $-CO-N$  $N-CH_2$ group gave rise to the fragmentation ion at *m/z* 198. Based on the above GC—MS analysis, a mono-trimethylsilyl ether derivative of tiaramide was formed under the present reaction conditions.

The pooled serum extract of several dog samples obtained after an oral dose of 200 mg of tiaramide hydrochloride was derivatized and analyzed by GC—MS in a similar manner. The results show that the mass spectrum of tiaramide isolated from dog sera and the mass spectrum of synthetic tiaramide were identical. Based on these studies, the present GC method is considered specific for the assay of tiaramide in dog sera.

Linearity and reproducibility

For each day of sample analysis, a standard curve with seven concentrations was prepared. The regression equation, determined by linear regression analysis was used for the calculation of tiaramide concentrations in the unknown serum samples. Table I lists the regression equations for the standard curves obtained from four different days. The correlation coefficient of the composite standard curve was 0.992 indicating linearity for the range studied (0.1—6.0 μg/ml).

The values for the mean relative area ± standard deviation (S.D.) and the coefficient of variation (C.V.) for each concentration point from four separate runs are shown in Table II. The average coefficient of variation for all con-

TABLE I

PARAMETERS OF REGRESSION EQUATIONS FOR DAILY AND COMPOSITE STANDARD CURVES

Standard curve	Slope	y-Intercept	Correlation coefficient
A	0.9974	0.0660	0.998
B	0.8806	0.1384	0.993
C	1.0028	0.0205	0.990
D	0.9931	0.1403	0.993
Composite	0.9601	0.0917	0.992

TABLE II

REPRODUCIBILITY OF EACH CONCENTRATION POINT
Data from standard curves A, B, C and D.

Concentration (μg/ml serum)	*n*	Mean relative area ± S.D.	C.V. (%)
6.0	8	5.8734 ± 0.5806	9.9
4.0	8	3.9607 ± 0.3490	8.8
2.0	8	2.0698 ± 0.1585	7.7
1.0	7	1.1082 ± 0.0729	6.6
0.4	8	0.4631 ± 0.0382	8.2
0.2	8	0.2268 ± 0.0176	7.8
0.1	8	0.1052 ± 0.0108	10.3
		Average C.V. (%)	8.5 ± 1.3

centration points indicating the day-to-day variations for a period of approximately two weeks was 8.5 ± 1.3%.

Application

A trial study to demonstrate the serviceability of the method was conducted in three male beagle dogs weighing 9.5—12.2 kg. An experimental tablet containing 200 mg of the active ingredient was orally administered to the fasting dogs. Fig. 3 shows the composite serum profile of the three dogs.

In another trial study, three human subjects were given an oral solution of 250 mg of RHC 2592. The serum samples were processed by the same procedure with the exception that 2.0 ml of the organic extract was taken in order to increase the assay sensitivity. As shown in the composite human serum profile (Fig. 4), the method was sufficient to assay the 8.0-h samples at 0.06 μg/ml.

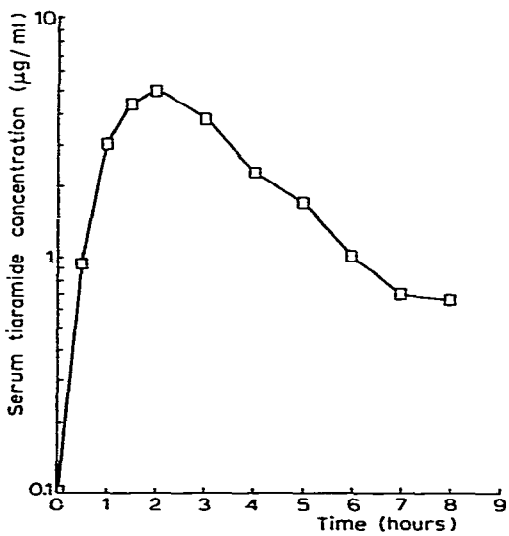

Fig. 3. A composite serum concentration—time profile of three dogs which had received an oral dose of an experimental tablet (200 mg RHC 2592).

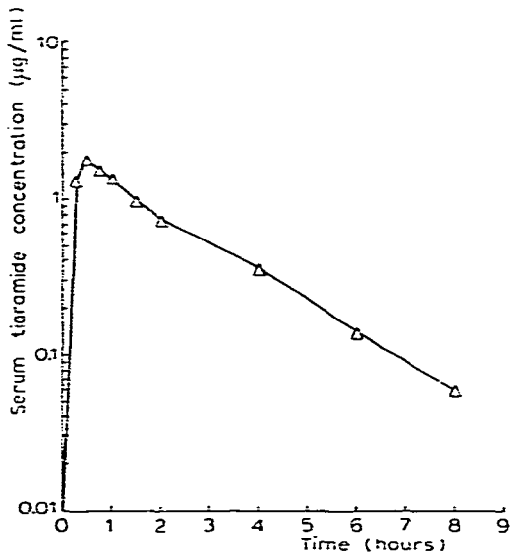

Fig. 4. A composite serum concentration—time profile of three human subjects who had received an oral solution of 250 mg RHC 2592.

CONCLUSION

The present method, as compared to the method reported previously, employed a simpler single extraction. The sensitivity was 0.1 μg/ml when an aliquot of one-tenth of the total extract was used for the assay. With a larger aliquot, as shown in the trial study with human subjects, a greater sensitivity can be attained if required.

Experience in this laboratory in the assay of unknown samples from bioavailability studies demonstrated that the combination of the simplified extraction procedure and the use of an auto-sampler has greatly increased the assay capacity; 60 to 70 unknown samples can be assayed in one man-day.

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