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# DETERMINATION OF 1-β-D-RIBOFURANOSYL-1,2,4-TRIAZOLE-3-CARBOXAMIDE (VIRAZOLE) IN BLOOD AND URINE BY CHEMICAL IONIZATION–MASS FRAGMENTOGRAPHY

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## SUMMARY

A combined gas chromatographic–mass spectrometric technique is described for the quantification of virazole in serum and urine. Proteins are removed by molecular filtration, lipids by extraction with dichloromethane and interfering endogenous constituents by acidic and basic ion-exchange resins. Virazole is quantified by monitoring the protonated molecular ions of the fully silylated derivatives of virazole (*m/e* 533) and the arabinose analog (internal standard) obtained by methane chemical ionization. The detection limit is 150 pg ($0.6 \cdot 10^{-12}$ mole) of virazole injected. In serum 10 ng/ml ($4 \cdot 10^{-8}$ mole) can be detected, 25 ng/ml quantified. In urine 0.5 μg/ml can be quantified without preconcentration. Virazole was detected in serum for at least 96 h at the 70-ng/ml level.


## INTRODUCTION

Structural analogs of naturally occuring purines and pyrimidines as inhibitors of *de novo* nucleic acid synthesis have been logical candidates in rational searches for antiviral drugs. Virazole (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, also known as Ribavirin) is a synthetic nucleoside structurally related to the purine biosynthetic precursor AICAR (5-amino-1-β-D-ribofuranosylimidazo-4-carboxamide). Virazole ($C_8H_{12}O_5N_4$, MW = 244.2), synthesized by ICN Pharmaceuticals (Irvine, Calif., U.S.A.)[1], is a stable, colorless crystalline compound, readily soluble in water, slightly soluble in ethanol. In tissue culture virazole exhibited activity against some twenty RNA and DNA viruses as determined by a variety of inhibition tests[2]. *In vivo*, virazole was shown to be effective when administered orally[3], intraperitoneally[4], by aerosol routes[5] in mice with experimental influenza infection, and when applied topically to herpes and vaccina virus-induced eye infection in rabbits[5]. In humans, moderate prophylactic doses given orally showed marginal effectiveness against chal-

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lenge influenza B virus infection[7] but no effectiveness against influenza A[8]; however, at higher doses (100 mg per day in four divided doses) virazole prevented moderate-to-severe illness in response to influenza A virus challenge[9]. Virazole has been licensed for human use in several countries in South America. In the U.S.A. there have been Phase I studies[10], and there is a current interest in clinical trials involving herpes viruses. Questions of teratogenicity in rodents are being investigated[11,12]. Teratogenic studies in primates have been negative[14]. A list of over 150 publications on various studies involving virazole was compiled by ICN Pharmaceuticals[13].

The mechanism of antiviral action of virazole is presently believed to be due to virazole 5'-triphosphate which selectively inhibits influenza viral RNA polymerase *in vitro*[19], while not inhibiting eukaryotic DNA polymerase, and eukaryotic RNA polymerase I and II or eukaryotic poly A polymerase[20] in a comparable *in vitro* system. The drug is enzymatically converted to virazole 5'-monophosphate inside the cells[15]. The metabolism of virazole has been studied in the rat: virazole-5'-monophosphate, 1,2,4-triazole-3-carboxamide, 1-$\beta$-D-ribofuranosyl-1,2,4-triazole-3-carboxylic acid, 1,2,4-triazole-3-carboxylic acid were identified by mass spectrometry (MS) and the di- and tri-phosphate metabolites by various forms of chromatography[16]. Metabolic and pharmacokinetic studies in humans are in progress in our laboratories.

The objective of the present work was to develop a specific and sensitive analytical technique for the quantification of intact virazole in serum and urine at therapeutic dose levels so that correlations between blood levels and toxicity as well as therapeutic efficacy may be established in clinical pharmacological trials. This report details the method developed.

Quantification of virazole in serum and urine was attempted by straight gas chromatography (GC)[17]; however, even a rather involved sample purification scheme was not adequate to provide specificity at low levels. Since virazole has only a modest ultraviolet absorption at about 225 nm (and virtually none at 254 nm), high-pressure liquid chromatography did not appear to be a good choice for trace analysis. The present technique is based upon the monitoring of the protonated molecular ions, $(M + 1)^+$ or quasimolecular ions, of the trimethylsilylated derivatives of virazole and the ara-analog of virazole (internal standard) formed upon chemical ionization with methane reagent gas in the ion source of the combined gas chromatograph–mass spectrometer–computer system. Since chemical ionization yields highly abundant $(M + 1)$ ions, the technique is inherently specific as well as sensitive.

## EXPERIMENTAL

### *Drugs and reagents*

Pure virazole, the ara-analog of virazole, and ($^3$H)-virazole (100 $\mu$Ci/$\mu$mole) were obtained by courtesy of ICN Pharmaceuticals. Synthesized samples of virazole metabolites, such as the free base, free acid and the monophosphate, were generous gifts from Dr. J. Witkowski of ICN Pharmaceuticals. All silylating reagents, including N,O-(bis)trimethylsilyltrifluoroacetamide + 1% trimethylchlorosilane (BSTFA + 1% TMCS), silylation grade pyridine and all GC column materials were purchased from Pierce (Rockford, Ill., U.S.A.). All solvents used were of nanograde quality (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.), all other chemicals

were of highest purity commercially available, and used without further purification. Dowex 50 and Bio-Rex 5 resins were from Bio-Rad Labs. (Richmond, Calif., U.S.A.). Centriflo membranes were from Amicon (Lexington, Mass., U.S.A.); these materials were prepared for use following the procedures recommended by the manufacturers. GC carrier gases and reagent gases for chemical-ionization MS were of high purity grade (Matheson, Rutherford, N.J., U.S.A.).

*Instrumentation*

Combined GC–low resolution MS was performed using a Finnigan Model 3300 instrument system (quadrupole-type mass analyzer) connected to a Model 6000 interactive data system and equipped with a chemical-ionization source and a capability for selected ion monitoring (mass fragmentography). High-resolution mass spectra were obtained with a JEOL Model JSCM-1 double-focusing instrument (magnetic-type mass analyzer) combined with a Hewlett-Packard gas chromatograph (Model 7620A).

*Internal standard*

The arabinose-analog of virazole was utilized as internal standard. The pure compound was dissolved in water to provide a 0.5-$\mu$g/$\mu$l stock solution. In all cases, adequate amounts of internal standard were added at the beginning of an analysis to yield a final concentration approximately in the middle of the expected concentration range of virazole.

*Preparation of standards and calibration samples*

Pure virazole and ara-virazole were dissolved in a mixture of BSTFA + 1% TMCS and silylation grade pyridine (1:1) in a PTFE-disk covered vial and allowed to stand at room temperature for 5–10 min. Solutions were made containing virazole in the range from 500 pg to 500 ng per sample injection (4 $\mu$l), and a known amount of internal standard was added to each sample. These samples were used to establish both low- and high-resolution mass spectra for the authentic compounds under various experimental conditions, to determine detection limits for the pure compounds, to establish sensitivities (in terms of computer-generated peak areas per unit sample quantity introduced) and also for daily routine mass range calibration.

To establish optimal analytical conditions and, subsequently, to obtain calibration curves, normal pooled human serum and urine samples were spiked with known quantities of virazole (using stock solutions made with methanol) and handled in the same manner described below for samples from experimental animals or human subjects. To compensate for irreproducible experimental errors, a full set of calibration runs with spiked serum or urine was made with every set of biological samples. The concentration range of the calibration standards covered the entire range of concentrations expected in the samples. The amount of internal standard was the same for all samples and standards analyzed. A blank sample, *i.e.*, with no virazole added, was also included in the calibration runs.

*Preparation of serum samples*

Blood samples for virazole were drawn from the contralateral arm at various intervals according to the particular protocol followed in a given series of clinical

investigations. The blood was allowed to clot at room temperature for about 25 min, the serum was separated by centrifugation at approximately 500 *g* at room temperature for 10 min, and stored at −80° until used. When samples from experimental animals were used, the handling was the same as described; when needed, samples were pooled from 2–5 mice treated in the same manner.

The first step in sample preparation was the addition of an adequate amount of internal standard to 2 ml of serum sample, followed by vortexing for a few seconds. Next, the serum was diluted with 2 ml of distilled and deionized water and again vortexed for a few seconds. The diluted serum was deproteinized by molecular filtration through membranes of 50,000 dalton exclusion limit (Centriflo, Amicon) which were prepared by presoaking in distilled water. Centrifuging at 1000 *g* (3000 rpm using a DuPont Model SS4 centrifuge) for 45 min at room temperature filtered more than 90% of the diluted serum. The clear filtrate was transferred into a culture tube and its volume was measured when needed. Next, the pH of the filtrate was adjusted to approximately 5–6 with the addition of 2–3 drops of 0.5 *N* HCl. To remove lipids, 3.0 ml of dichloromethane was added, the mixture first vortexed for a few seconds and then centrifuged at 780 *g* (2500 rpm) at room temperature for 15 min. Next, the aqueous (upper) layer was removed into another culture tube.

A number of interfering constituents were removed by passing the deproteinized and lipid-extracted samples through an ion-exchange resin bed consisting of a Dowex 50 (acidic) and Bio-Rex 5 (basic) resins. Individual ion-exchange columns were prepared using disposable Pasteur pipettes plugged at the tapered end with a small amount of glass wool. A 3-cm long bed of Bio-Rex 5 resin (100–200 mesh, chloride form) was then slurry packed and allowed to settle by gravity. On top of this was placed a 3-cm long bed of Dowex 50 resin (100–200 mesh, hydrogen form), also allowed to settle by gravity. It is noted that no special efforts were made to exactly duplicate resin densities and/or bed heights beyond reasonably careful laboratory work. The aqueous sample was filtered through this bed system at atmospheric pressure by placing it on top and letting it drip through. This procedure took only a few minutes. The filtrate was placed in a 20-ml vial, shell-frozen in a dry-ice–acetone bath and lyophylized overnight; the dry product was a whitish light flaky powder. Prior to MS analysis, the samples were silylated by adding 100–200 $\mu$l BSTFA + 1% TMCS–pyridine (1:1) and letting the samples stand at room temperature for 5–10 min.

*Preparation of urine samples*

Urine samples from patients or control pooled urine samples were stored frozen without adding any kind of preservative. The first step in sample preparation was the addition of an adequate amount of internal standard to 0.2 ml urine, followed by vortexing for a few seconds. These samples were added dropwise with stirring to 3 ml of absolute methanol. The mixture was next centrifuged at 10,000 *g* (17,000 rpm in a Sorvall Model RC-3 centrifuge) at 10° for 20 min. The liquid phase was next extracted once with 2 ml of *n*-hexane, the hexane layer discarded, and the methanol layer evaporated to dryness with a gentle stream of dry nitrogen in a water bath kept at 50°. Prior to MS analysis, the samples were silylated by adding 200 $\mu$l of BSTFA + 1% TMCS–pyridine (3:1).

*Gas chromatography–mass spectrometry (low resolution)*

For GC separation a glass column (1 m × 2 mm I.D.) filled with 3% OV-17 or 3% SE-30 on Chromosorb W HP (80–100 mesh) was employed. Injector temperature was kept at 260°. There was no separator between the gas chromatograph and the mass spectrometer; the connecting tube was kept at 240°. Methane gas was used both as the GC carrier gas and as the reagent gas in the chemical-ionization source of the mass spectrometer.

For the analysis of virazole in serum, the GC column was temperature programmed from 175 to 240° at a rate of 8°/min; urine samples were analyzed isothermally at 230°. To remove other constituents present in biological samples, and also for periodic cleaning, the column was heated to 270° until no more effluent could be detected.

After introducing a sample, normally 4 µl, into the gas chromatograph, the effluent was vented for 0.5 min to avoid contamination of the ion source with the extra silylating reagent and pyridine. The quadrupole mass spectrometer was operated in the chemical-ionization mode using methane (1 torr pressure) as the reagent gas. Operational parameters were adjusted daily using pure compounds to obtain maximum sensitivity. Chemical-ionization mass spectra were obtained by operating the instrument in the "full scanning" mode. For quantification, the instrument was operated in the "selected-ion monitoring" mode, recording ions characteristic of the compounds being analyzed: *m/e* 533, the $(M + 1)^+$ ion of both virazole and ara-virazole and *m/e* 517, corresponding to the loss of a $CH_3$ group. It is noted (see Discussion) that in the case of urine there was interference from endogenous constituents at *m/e* 517; thus, this peak, although monitored, is of little significance. At any rate, only the $(M + 1)^+$ ions were needed for quantification; the other ions were monitored mainly to confirm the presence of virazole.

*Quantification*

An individual calibration curve was obtained with every series of samples analyzed. In these curves the observed ratio of the peak area of virazole to that of ara-virazole was plotted against the known concentration of virazole used for spiking. These data points yielded a straight line within the limits of reproducibility.

*High-resolution mass spectrometry*

For complete identification of both the fully silylated and dehydrated virazole (see Discussion), initial experiments were made using combined GC–high-resolution MS. The combined system was operated in a routine manner using electron-impact ionization at 70 eV. Photoplate detection was employed using Ilford Q-2 or silver-bromide type (Ionomet) photoplates. Line position (masses) and intensities were determined with computerized microdensitometry. All masses were determined to an accuracy of 3–5 millimass units.

## RESULTS AND DISCUSSION

*Mass spectra of virazole*

When silylated virazole was introduced via a solid probe accessory directly into an electron-ionization source (70 eV), the mass spectrum contained a weak, but

readily measurable, molecular ion at *m/e* 532. High-resolution mass measurements confirmed that the peak corresponded to the uptake of four trimethylsilyl groups. Thus silyl incorporation occurs at all the sugar hydroxyls and also the amino function which becomes monosilylated. When silylated virazole was introduced into the ion source after passing a GC column and also a jet-type helium separator, the fully silylated molecular ion did not appear. Instead, an ion of nominal mass *m/e* 442 was detected. High-resolution mass measurement (accuracy to $\pm$2–3 millimass units) revealed a composition of $C_{17}H_{34}N_4Si_3$, indicating the formation of a cyano-compound and the uptake of only three trimethylsilyl groups. The electron-ionization mass spectra of silylated virazole were similar using both magnetic and quadrupole type mass analyzers. The formation of the dehydrated compound was not affected by the removal of the jet-separator; the "high-pressure" helium electron-ionization spectra also exhibited the ion at *m/e* 442 (Fig. 1). This dehydration step clearly takes place in the injector port of the gas chromatograph, and the degree of dehydration depends upon the temperature of the injector port (see next section). Desilylation by hydrolysis in GC injection ports has also been observed by other investigators[18].

The chemical-ionization (methane) mass spectrum of the fully silylated virazole molecule (Fig. 2) reveals highly abundant protonated molecular, $(M + 1)^+$, as well as $(M - 15)^+$ ions, both of which are suitable for selected-ion monitoring.

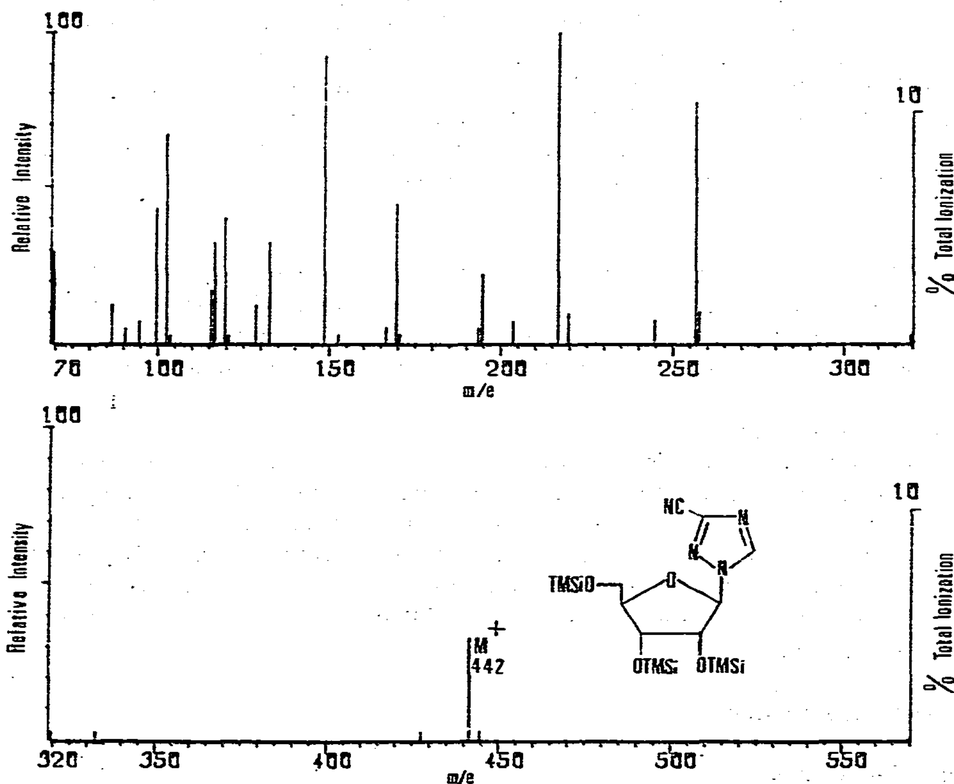


Fig. 1. Electron-impact mass spectrum of the trimethylsilyl derivative of virazole (75 eV). The derivative was injected via a gas chromatograph injector port where dehydration occured.

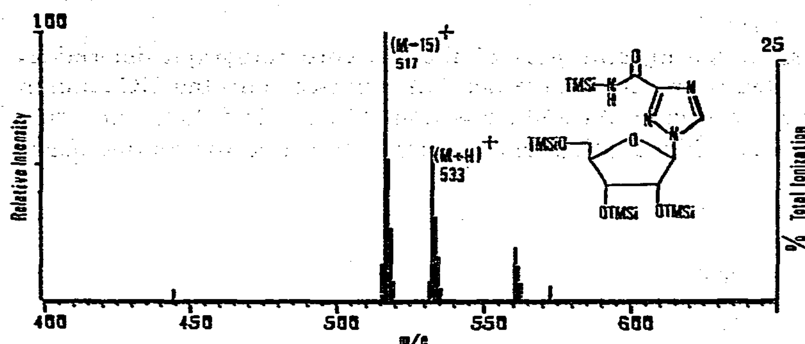


Fig. 2. Chemical-ionization (methane) mass spectrum of the fully trimethylsilylated derivative of virazole. No dehydration in the gas chromatograph injection port.

The mass spectrum of ara-virazole is virtually identical to that of virazole (Fig. 3). The retention times are sufficiently different to permit differentiation (see below). Chemical-ionization mass spectra can also be obtained for the 1-ribosyl-5-cyano-1,2,4-triazole dehydrated product (Fig. 4).

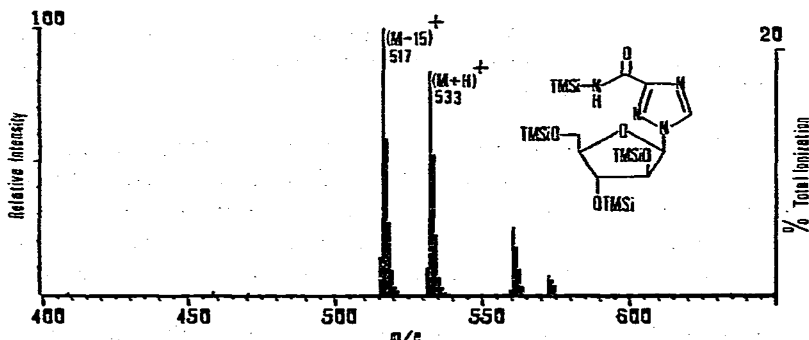


Fig. 3. Chemical-ionization (methane) mass spectrum of the fully trimethylsilylated derivative of the arabinose analog of virazole.

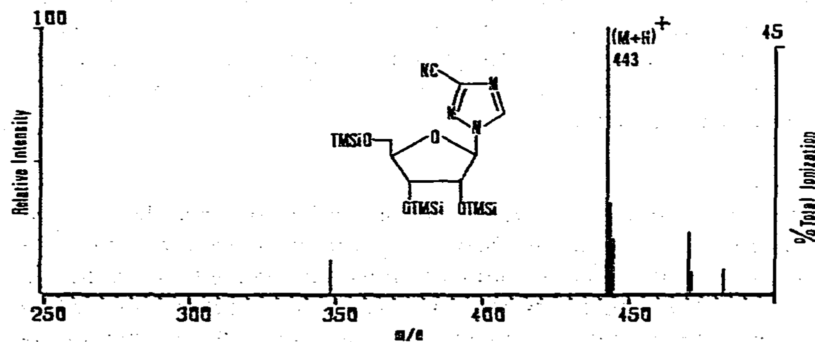


Fig. 4. Chemical-ionization (methane) mass spectrum of silylated virazole showing dehydration.

*Gas chromatography of virazole*

The temperature of the injector port of the gas chromatograph determines the ratio of the dehydrated to intact virazole derivative passed onto the GC column and eventually into the ion source. At 260°, less than 5% of virazole is converted into the dehydrated product (Fig. 5); this temperature is best used for routine quantification analyses.

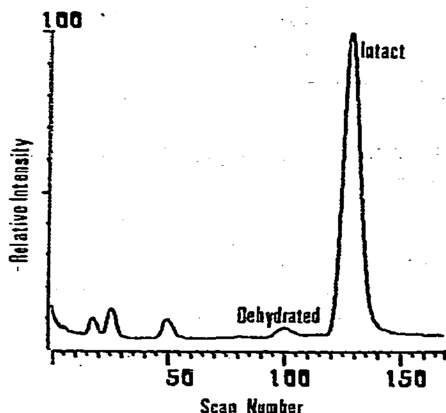


Fig. 5. Total-ion monitogram showing that intact fully trimethylsilylated virazole can be passed through the gas chromatograph using the conditions given in the text. The amount of dehydrated virazole formed is less than 5% of the total injected.

Either SE-30 or OV-17 (3%) may be used as GC column material. With the 3% SE-30 columns the virazole peaks appear at 5–10° higher temperature than in the case of OV-17. In most cases a column length of 1 m was employed, however, equally good results were obtained with 2-m long columns. Temperature programming from 175–240° at a rate of 8°/min permitted adequate separation of virazole and the ara-analog.

Methane served both as GC carrier gas and reagent gas in the chemical-ionization source. The GC properties of the silyl derivatives of virazole and its ara-analog were similar with both methane and helium carrier gases, however, retention times were longer when methane was employed.

The GC retention times of virazole and the ara-analog are 1.4 and 1.9 min, respectively. Although these retention times are reproducible as long as experimental conditions are kept unchanged, precise knowledge of retention times is not essential since single ions are monitored at two masses and the mass fragmentograms (see below) reveal the relevant peaks clearly.

*Detection limits and quantification*

The limit of detection of pure virazole is 150 pg, or $0.6 \cdot 10^{-12}$ mole underivatized weight injected into the combined gas chromatograph–mass spectrometer system. Limit of detection is defined as the amount of substance needed to produce a signal-to-noise ratio of 2:1. For quantification one needs to inject about 300 pg virazole so that peak areas can be determined by the computer after proper background correction.

In serum (human, rat, or mice) the limit of detection of free circulating virazole is 10 ng/ml or $4 \cdot 10^{-8}$ mole. Fig. 6 shows selected-ion monitoring (mass fragmentogram) if virazole and ara-virazole (internal standard) in a human serum sample. The amount of virazole added to this calibration sample was 100 ng/ml. It is clear that one-quarter of the virazole peak can easily be quantified by the computer, thus virazole can be quantified in serum to 25 ng/ml, or $1 \cdot 10^{-7}$ mole. This is adequate for all expected pharmacokinetic investigations.

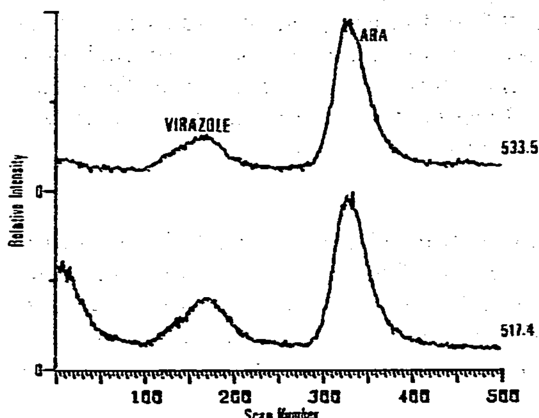


Fig. 6. Selected-ion monitoring of 100 ng/ml virazole in human serum, also showing the ara-virazole analog used as internal standard.

In urine, quantification of virazole is possible to 0.5 $\mu$g/ml with the simple sample preparation technique described. Fig. 7 shows selected-ion monitoring of virazole in a urine calibration sample containing 5 $\mu$g/ml virazole. If needed, the lower level can be extended by as much as a factor of 10 using preconcentration; however, it is not expected to be important to quantify virazole in urine in trace quantities. It is noted, that there is an interference in urine at *m/e* 517 (Fig. 7) with the same

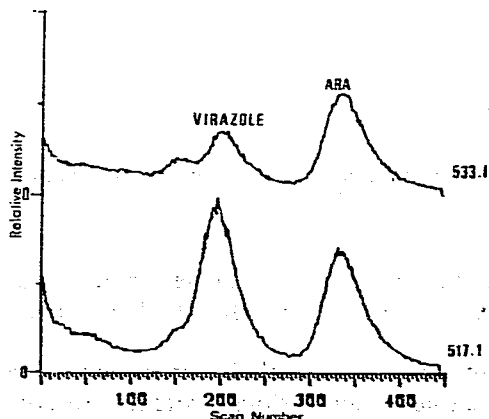


Fig. 7. Selected-ion monitoring of 1 $\mu$g/ml virazole in human urine, also showing the ara-virazole analog used as internal standard.

retention time as that of virazole. This is unimportant since that peak (though monitored to confirm the identity of the ara-analog) is not used in the process of quantification. There is no interfering peak at the *m/e* 533 trace, the one used for quantification.

The quantification of virazole is accomplished with the aid of calibration curves. These are obtained by plotting the ratio of the areas of virazole and ara-virazole (as determined by the computer) against the known quantity of virazole added to a particular calibration sample. The same quantity of internal standard was added to all samples. The calibration curves thus obtained were straight lines, within a concentration range of a factor of 100 and intercepted the $y$-axis at or very near the origin. Reproducibility measurements were made ($n = 4$–$6$) for each concentration point on the calibration curve in the 50-ng/ml to 5-$\mu$g/ml range for serum and the 1–10 $\mu$g/ml range for urine. The coefficients of variation (defined as standard deviation divided by the mean $\times 100$) were in the 5–15% range.

*Applications*

Fig. 8 shows the results of the quantification of intact virazole in the serum of a patient who received 200 mg/m$^2$ virazole. The first part of the curve indicates a sharp drop in virazole content with a first half-life of the order of 15–20 min. It is noted that virazole remains in the serum for a considerable period of time. For example, the point shown on the curve at 96 h corresponds to 70 ng/ml, well above the limit of detection.

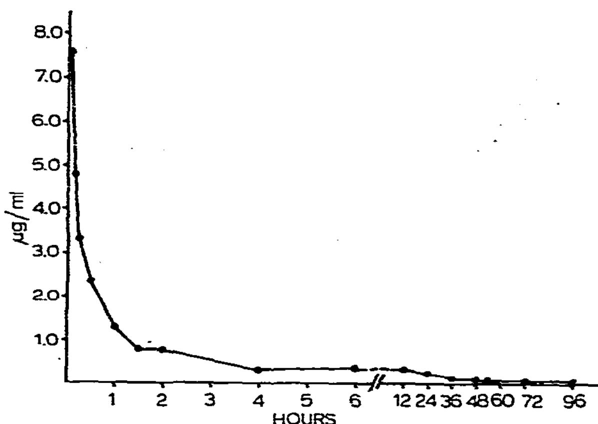


Fig. 8. Blood levels of virazole, using 200 mg/m$^2$ dose. The level at 96 h is 70 ng/ml, considerably above the limit of detection.

When higher doses of virazole were administered orally, the serum levels of virazole increased. The persistence of virazole in serum for at least 96 h has been repeatedly observed.

The techniques developed for the quantification of virazole in serum and urine are now being applied to patients receiving virazole under various protocols to correlate pharmacokinetic data and clinical observations. Also in progress is work to establish the site of virazole pools responsible for the persistence of the drug in serum.

ACKNOWLEDGEMENTS

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