Determination of Small Amounts of Thiacetazone® in Serum

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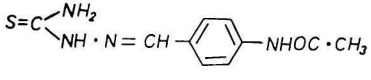
A modification of the method described in the literature for Thiacetazone determination in blood serum by means of UV spectrophotometry is presented together with an evaluation of its results. Quantitative data are obtained from absorbance values read off at 330 nm, but an absorption curve in the 310—340 nm range of wavelength is prepared for each sample separately. A correct evaluation of the absorption curve enables results to be obtained even with a Thiacetazone serum concentration below 0.5 μg/ml and in addition, it permits artefacts caused by the absorbance of interfering agents, to be detected. The method is quick, without great technical exigencies and at the same time sensitive and reproducible.

A control over the fate of administered Thiacetazone still presents a problem. A follow-up of the complex mechanism of action demands, before all else, an exact knowledge of the resorbing and the releasing conditions that are involved; in addition, the existing routine procedures of analysis are inadequate to express the exacting quantitative relationships.

Various methods have been proposed and applied to determine Thiacetazone levels in extra-cellular fluids and in certain organs. The determination is, however, rather difficult by the fact that following the usual application dose (approximately 2.5 mg/kg b. w. per day), the drog level in the serum varies between 0.01 and 0.25 mg%, *i.e.* 0.1 and 2.5 μg/ml.

As apparent from the structure proper of Thiacetazone — a *p*-acetylaminobenzaldehyde thiosemicarbazone:



— its chemical determination may be based on the reactivity of the two functional groups, namely:

1. the acetylamino group,
2. the thiosemicarbazone group.

The principle of determination in the first case consists in hydrolytic splitting of the molecule, releasing the amino group which is determined to be a colour reaction following diazotization and copulation [1].

Methods based on the diazotization principle have been evaluated and discussed by *Sturm* and *Wernitz* [2]. Nevertheless, the fact remains that this reaction is also raised by other aromatic amines present in biological materials, so that they are not specific.

Procedures attempting to eliminate the interfering substances by repeated extractions of Thiacetazone [3] introduce further errors into the determination. Moreover, losses of material are considerable and recoveries amount to no more than about 50%.

Methods utilizing the thiosemicarbazone group instead of acetylamines, are applied directly without any preceding hydrolysis. Such are, for instance, the relatively little known methods described by *Wollenberg* [4] in which a reaction occurs between ω-halogenoacetophenone and thiosemicarbazide on thiazole, and whose yellow staining can be colorimetrically measured. The reaction is undoubtedly specific, but the procedure has not passed into current practice as ω-halogenoacetophenones are strongly irritant the eye conjunctiva.

Another method [5] involves the production of a blue coloured complex between the =C=S group and nitroprusside under the oxidizing action of brome. The reaction develops in the presence of heat. The sensitivity of the method, however, is rather limited.

A highly specific method for Thiacetazone, described by *Spinks* [6], is based on the principle of characteristic absorption in the UV spectrum following the extraction on chloroform. This method has been modified by *Wernitz* and *Tornus* [7] who determined Thiacetazone in chloroform—amyl alcohol extract (Fig. 1). Their modified procedure has been taken as our starting point and we have used it, with some modification, in all our thiacetazone assays in serum in case of very low concentrations. Thiacetazone levels have been determined in the serum of human individuals who were given 150 mg of the drug daily, perorally, in a long term treatment.

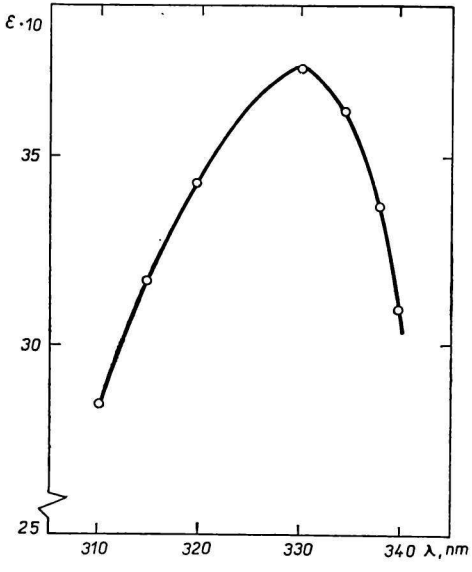


Fig. 1. Thiacetazone absorption curve in an chloroform—amyl alcohol mixture, 4 : 1. Thiacetazone concentration 5 μg/ml, measured against a pure solvent in 20 mm absorption cells.

Experimental

Materials and instrumentation

Sec. sodium phosphate solution (Na_2HPO_4), cold saturated.
Chloroform, spectrally pure, Merck.
Amyl alcohol, anal. grade, Lachema.
Separating funnels, 50 ml.
Filter paper, Whatman chromatographic No. 1.
Spectrophotometer, Beckman DU.
Absorption cells, UV grade silica, 20 mm.

Method

Freshly-drawn blood was immediately centrifuged. The levels were determined at zero hour, then at 40 minutes, 1, 2, 3 and 6 hours following administration of the drug. The samples were processed within 8 hours after collection and stored in the meantime, in a refrigerator at +4°C.

One ml of Na_2HPO_4 solution, 2 ml of serum and 10 ml of a chloroform—amyl alcohol mixture, 4 : 1, are dosed into a separating funnel. The contents are vigorously shaken for about 1 minute and then left to stand for another minute to allow the aqueous phase to

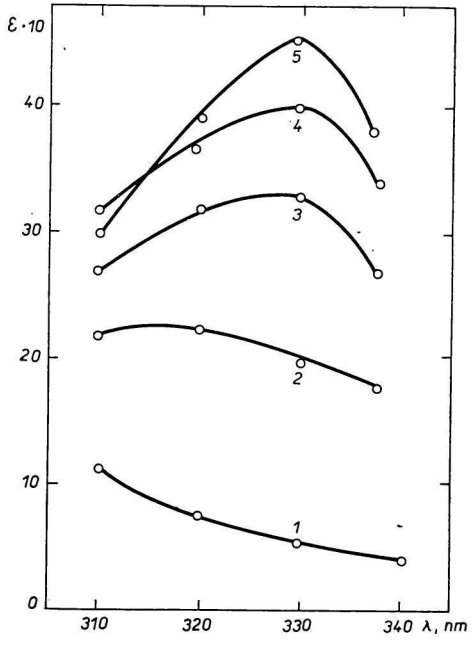


Fig. 2. Absorption curves of Thiacetazone released into the blood between 0 and 6 hours following application of 150 mg Thiacetazone per day.
1. before Thiacetazone administration; *2.* excretion after 40 minutes; *3.* excretion after 60 minutes; *4.* excretion after 180 minutes; *5.* excretion after 360 minutes.

separate completely from the chloroform—amyl alcohol extract. The organic phase is then passed through filter paper into a ground-glass test tube, and its absorbance measured on a spectrophotometer at the wavelengths of 310, 320, 330 and 340 nm, against a blank prepared similarly as the sample, except that instead of the serum, distilled water is used in 20 mm layer. The absorption curves obtained were evaluated. The amount of Thiacetazone present in the sample was calculated from the calibration curve, constructed from a standard Thiacetazone sample, added into the serum after extraction. The absorption values obtained at 330 nm are plotted on the calibration curve.

Results and Discussion

The method described is simple in application, technically not demanding, specific, and under replication, confirms the results reported by other authors. Yet in spite of adequate sensitivity and specificity, certain concrete problems were met when values below 0.5 μg/ml of serum had to be determined. As it is generally known, Thiacetazone is resorbed rather slowly, attaining its maximum excretion in approximately 6 hours after application. Serum levels are low 40 to 60 minutes following administration, sample absorbances range between 0.02 and 0.04 which corresponds (Fig. 2) to the concentration level below 0.5 μg/ml serum. However, this same absorbance is yielded also by numerous sera not containing any Thiacetazone at all (Fig. 3).

This disproportion could be eliminated by the use of a serum blank, but technically this is not possible except on the first day of the drog administration. The reason is

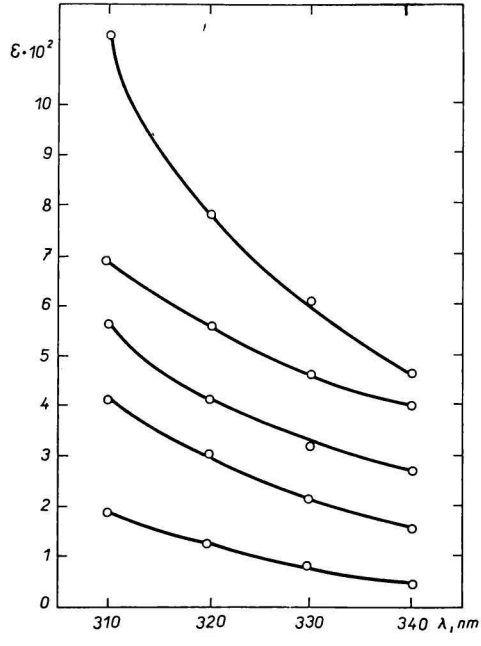


Fig. 3. Absorption spectra of various Thiacetazone free sera.
Extracting agent chloroform—amyl alcohol mixture, 4 : 1, measured against a chemical blank in 20 mm absorption cells.

that Thiacetazone disappears only slowly from the blood where its traces persist as late as 24 hours following application (Fig. 4). Thiacetazone free serum from another patient who did not receive this drug has proved unsatisfactory for nearly every blank showed an individual absorption: this in itself is no handicap when Thiacetazone serum concentration exceeds 1 μg/ml. This value is, however, always lower at the beginning of excretion. In order to reach a solution of the problem, absorption curves from every sample were prepared in the range of 310—340 nm,

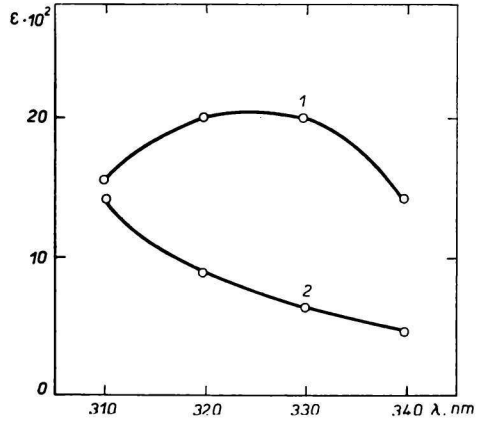


Fig. 4. Absorption curve of patient before Thiacetazone administration (*1*). Absorption curve of the same patient following intake of 150 mg Thiacetazone per day, 24 hours after application (*2*).

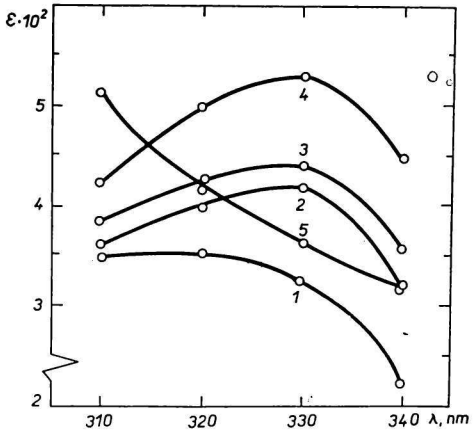


Fig. 5. Thiacetazone absorption curves.
1. serum extract following addition of 0.1 μg Thiacetazone in 1 ml; *2.* serum extract following addition of 0.25 μg Thiacetazone in 1 ml; *3.* serum extract following addition of 0.50 μg Thiacetazone in 1 ml; *4.* serum extract of patient receiving of Thiacetazone; *5.* serum extract of patient without of Thiacetazone.

and measured against a blank from the applied chemicals. Distilled water or pure solvents proved unsuitable for blanks. Sera containing even minor traces of Thiacetazone already give a typical absorption spectrum that differed markedly from those shown by Thiacetazone free sera, as evident in Figs. 3 and 5. Trace amounts of Thiacetazone are sufficient to alter the pattern of the curves from concave (Fig. 3) to convex (Fig. 5). Serum samples were evaluated by the shape of the measured curves: if convex curves were obtained, the presence of Thiacetazone traces was presumed (Fig. 5, curve *1—4*), in the opposite case, other interfering agents were involved (Fig. 5, curve *5*). Naturally, data on the quantity of Thiacetazone present are given by the values read up at 330 nm and are evaluated from the calibration curve, or calculated mathematically.

An analysis of the typical absorption curves proved helpful also in the evaluation of atypical cases of excretion. Since a protracted excretion is involved in the case of Thiacetazone, erroneous conclusions might be drawn in some cases from the measured absorbance values, as for instance, when the 40 minutes sample is analyzed. Artefacts may likewise be produced by other drugs taken by the patient. After processing a greater number of sera and analysing numerous absorption curves, one attains proficiency at routinely evaluating the curves and detecting artefacts. An introduction of the analysis of characteristic spectral curves will enable a safe determination of Thiacetazone even in concentrations below 0.5 μg/ml of blood serum.

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