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ACKNOWLEDGMENTS AND ADDRESSES

Received August 27, 1976, from the *Department of Chemistry, University of Massachusetts–Boston, Harbor Campus, Boston, MA 02125.*

Accepted for publication October 5, 1976.

Supported by Public Health Service Research Grant CA17436-01 from the National Cancer Institute.

The authors thank Dr. Andre Courtin and Dr. G. Muller for gifts of chemicals, Dr. John Clark for assistance in crystal growing, and the staff of the Research Computer Center for help.

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Simultaneous Automated Determination of Spironolactone Metabolites in Serum

PETER NEUBERT [x] **and KLAUS KOCH**

Abstract □ An automated two-phase method for the simultaneous fluorometric determination of the spironolactone metabolites canrenone (II) and canrenoic acid (III) in serum is described. The determination is performed by two dichloroethane extractions of the same serum sample at different pH values. The fluorescence developed in 65% (v/v) sulfuric acid is measured in two separate fluorometers (one each for canrenone and canrenoic acid). Comparable specificity and sensitivity to the manual procedure are obtained, with sensitivity limits of 20 ng of II/ml and of 30 ng of III/ml in serum. This method is applicable to the automated determination of drugs and metabolites in biological material when several extraction steps are involved.

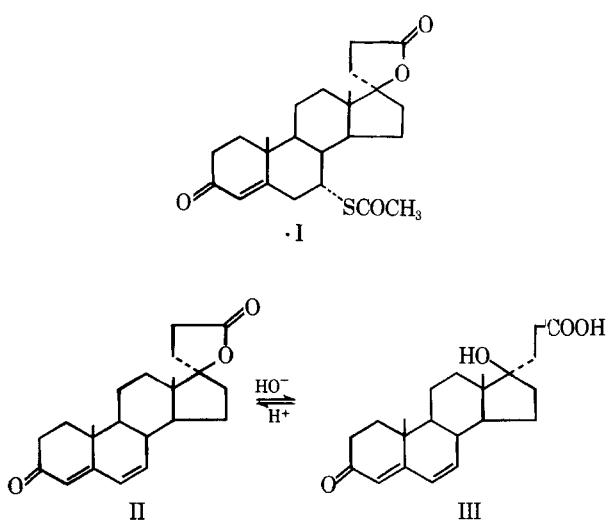
Keyphrases ◨ Canrenone and canrenoic acid—automated simultaneous fluorometric analyses, serum □ Fluorometry—automated simultaneous analyses, canrenone and canrenoic acid, serum □ Automated analyses—simultaneous fluorometric analyses of canrenone and canrenoic acid, serum □ Spironolactone metabolites—canrenone and canrenoic acid, automated simultaneous fluorometric analyses, serum ◨ Aldosterone antagonists—canrenone and canrenoic acid, automated simultaneous fluorometric analyses, serum

Spironolactone[1] (I) inhibits the aldosterone-initiated reabsorption of sodium ions from the distal portion of the renal tubule. The increased excretion of sodium ions is the underlying principle of the diuretic action in the treatment of edema.

Compound I is rapidly metabolized in the body after oral administration (1, 2). The elimination of the thioacetyl group on C-7 gives rise to canrenone[2] (II). Canrenone, as a γ-lactone, is in a pH-dependent equilibrium with the corresponding γ-hydroxycarbonic acid, canrenoic acid[2] (III) (Scheme I).

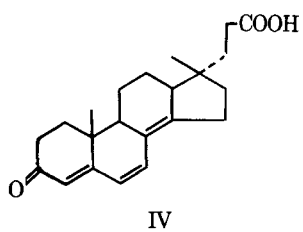
Compounds II and III are relatively stable in the physiological pH range of 5–9, but greater pH changes and enzymatic processes change the ratio of the two metabolites (3). For this reason, knowledge of the blood level curves for both metabolites is important for assessing the absorption, distribution, and excretion of spironolactone.

Gochmann and Gantt (4) first described the procedure for the fluorometric determination of canrenone by measurement of the fluorescence in 65% sulfuric acid. The structure of the fluorophore (IV) produced by the reaction of sulfuric acid with canrenone and canrenoic acid was elucidated (5).



Scheme I

The high intensity of the fluorescence of the trienone (IV) in 65% sulfuric acid permits the sensitive determination of the metabolites in both manual (6) and automated procedures. The manual procedure is time consuming since four extractions are necessary. The analysis is complicated further by the caustic nature of the reagents dichloroethane and 65% sulfuric acid. The automation of the analytical procedure was therefore desirable and is the subject of this report.



[1] Aldactone, Boehringer Mannheim GmbH.

[2] Compound II was formerly referred to as aldadiene, and III was referred to as aldadienic acid (1, 2).

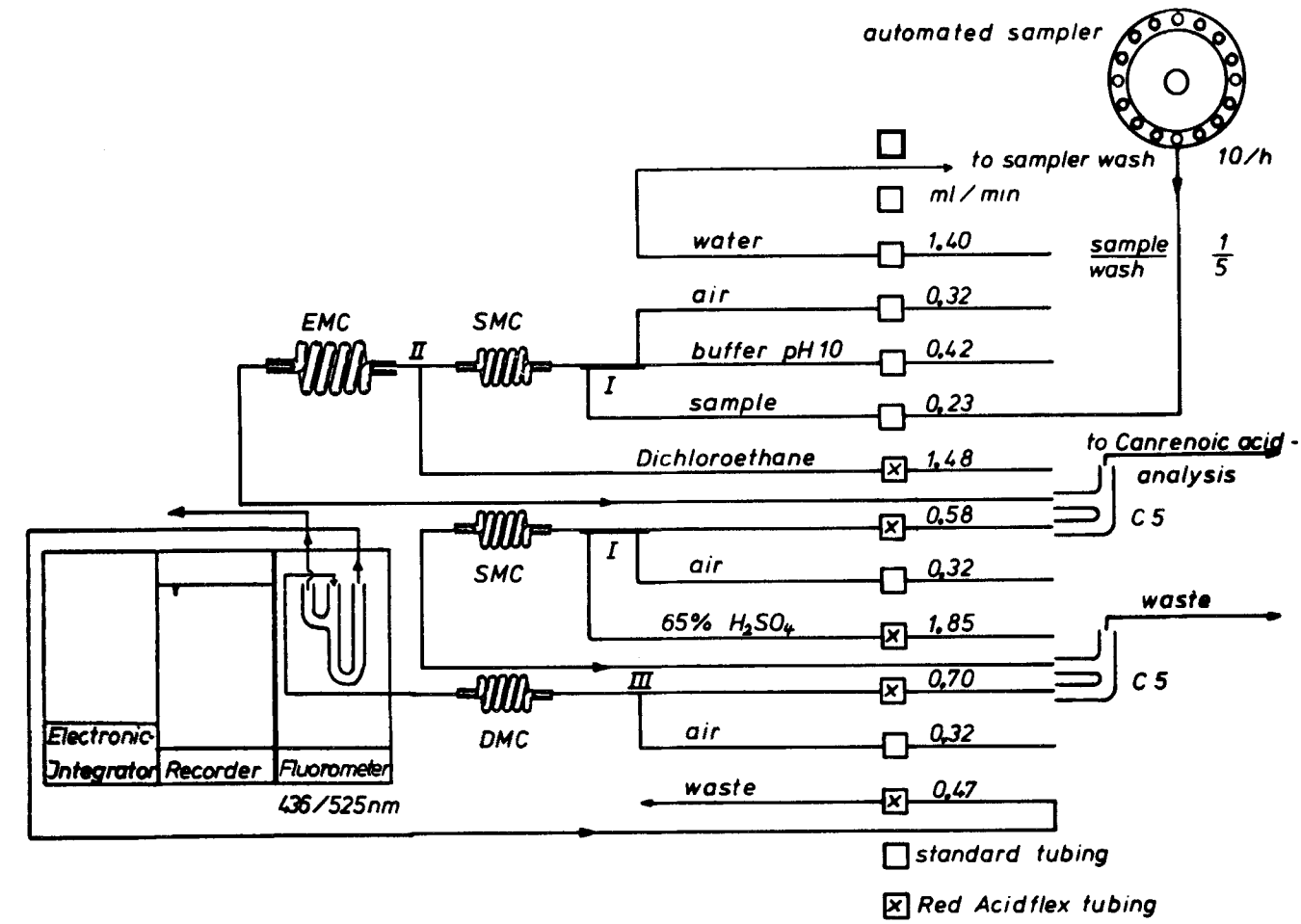


Figure 1—*Flow diagram for automated determination of canrenone. Key: SMS, single mixing coil; DMC, double mixing coil; EMC, extracting mixing coil; C5, phase separators; and I, II, and III, glass connectors.*

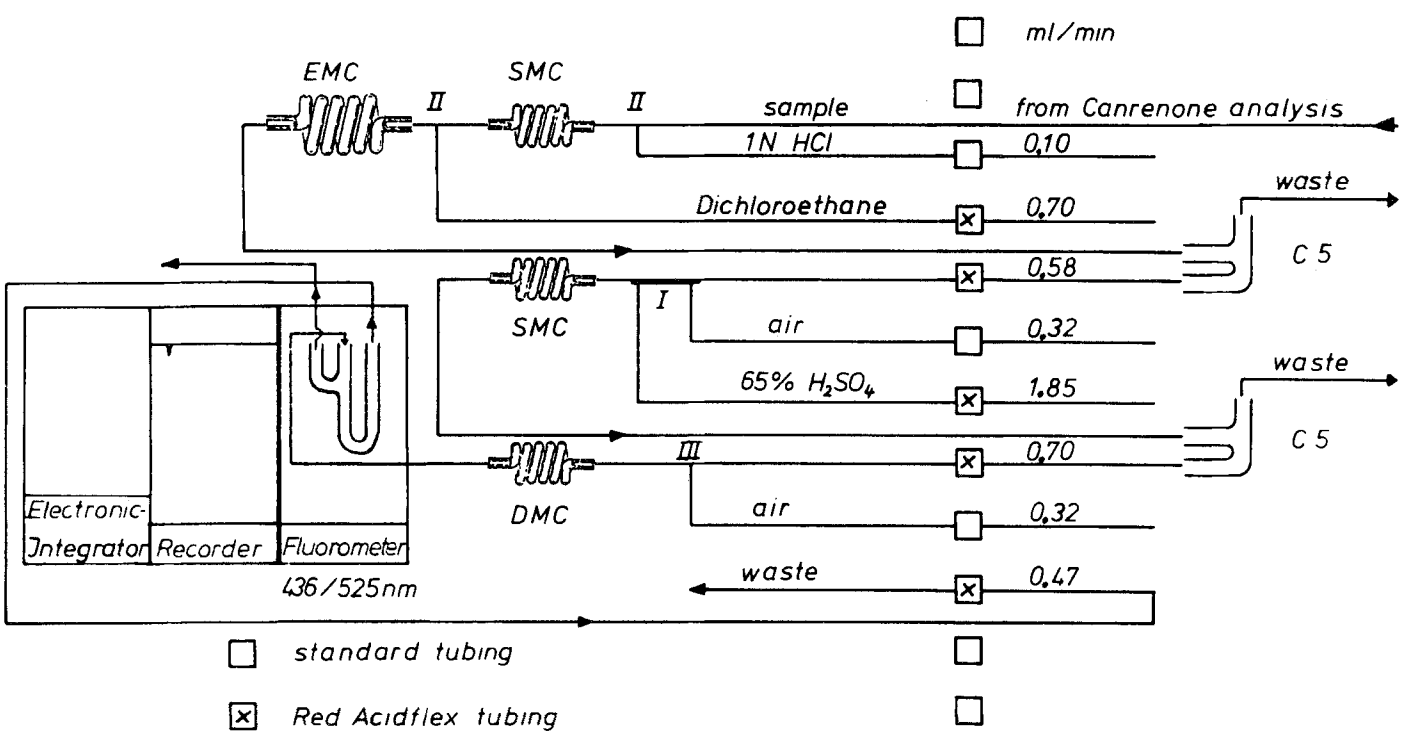


Figure 2—*Flow diagram for automated determination of canrenoic acid. Key: same as Fig. 1.*

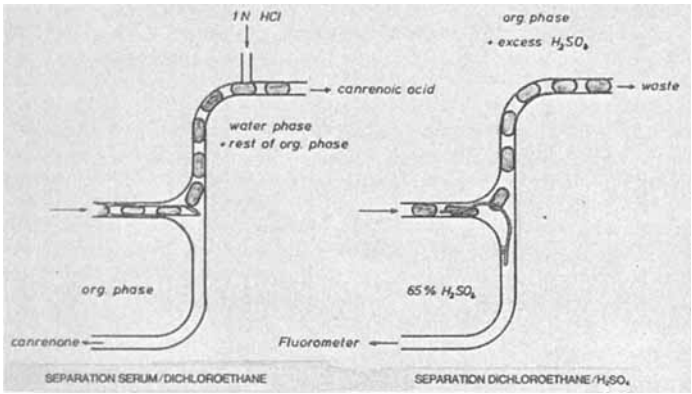


Figure 3—*Phase separation.*

EXPERIMENTAL

Apparatus—An automatic sampler and proportioning pump[3], two fluorescence spectrophotometers[4] with flowcells and a special cell holder designed to measure the fluorescence of 65% sulfuric acid in a continuous flow, a recorder[5], an electronic integrator[6], and a computer[7] were used.

Materials—The 0.04 *M* Britton–Robinson universal buffer (pH 10), 1 *N* hydrochloric acid containing 0.1 ml of wetting agent[8]/liter, dichloroethane containing 1.0 ml of wetting agent[8]/liter, and 65% (v/v) sulfuric acid (68 ml of 96% sulfuric acid plus 32 ml of water) were analytical reagent grade.

Standards—Separate stock solutions of canrenone and canrenoic acid, 500 mg/liter, were prepared in methanol. Working solutions were obtained by appropriate dilution with water. Suitable aliquots of the solutions were added to blank serum as standards.

Automated Analysis—The flow diagrams of the automated system for the determination of canrenone and canrenoic acid are shown in Figs. 1 and 2, respectively. The 19 pump tubes, however, only require one pump.

Canrenone—The sample was mixed in the predetermined volume ratio with previously air-segmented buffer to obtain a pH of 9. Dichloroethane was added as a solvent to give a two-phase system. The sample passed through an extracting mixing coil (EMC) followed by phase separation. The upper aqueous phase was led to the determination of canrenoic acid (Fig. 2). An aliquot of the organic phase was taken from below and reacted with 65% sulfuric acid. The organic phase was discarded, and an aliquot of the sulfuric acid phase was fed into the fluorometer. A delaying mixing coil (DMC) ensured an adequate reaction time.

The intensity of the fluorescence measured in the fluorometer was recorded. The corresponding peak height was determined with an electronic integrator[6], which could be used to print either peak areas or peak heights. The data were transferred to a punch tape and processed by means of an off-line programmed computer[7].

Canrenoic Acid—The simultaneous determination of canrenoic acid in the same serum sample ran in parallel but with a slight time delay. The serum sample was acidified with 1 *N* hydrochloric acid after removal of canrenone. This acid pH caused the lactonization of canrenoic acid to canrenone. The other steps were the same as those described for canrenone.

RESULTS AND DISCUSSION

The simultaneous determination of canrenone and canrenoic acid in the same serum sample with retention of the two-phase system requires four extraction steps and, correspondingly, four phase separations.

The separation of the phases takes place in small separators (Fig. 3) on the basis of the differing solvent densities and the differing wettability of the polytef tongue. Phase separation usually was facilitated with a small polytef rod. This technique also was used for the separation of dichloroethane and sulfuric acid. The separation of serum and dichloroethane, however, is more critical. In this case, optimal results were obtained by using a piece of polytef tubing (0.8 mm i.d.).

The best solvent for separation and extraction was dichloroethane because of the marked difference in density with regard to serum and sulfuric acid and because of the lower tendency to form emulsions in comparison with chloroform.

Tubings[9] made of material resistant to acid and solvents were used in the pump region for the transport of the solvents and sulfuric acid. All connecting pieces within the analytical unit were glass.

Phase separation and transport with low carryover were greatly facil-

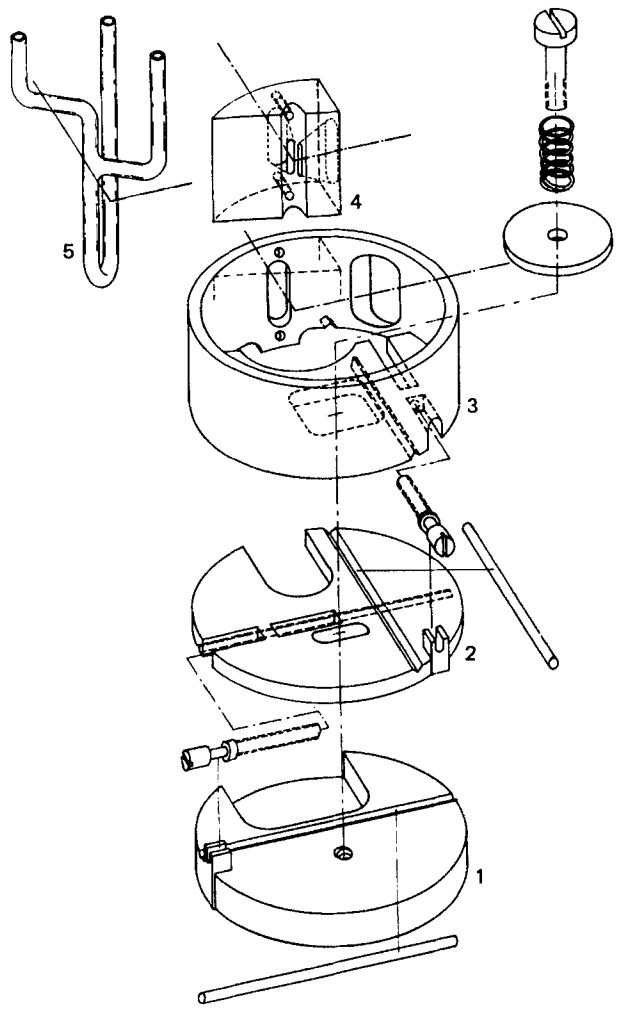


Figure 4—*Cell holder consisting of three brass plates. The ground plate (1) is made fitting to the base of the fluorometer cell compartment. The next plate (2) is adjustable by means of a screw in the direction of the excitation source. The other parts are the upper plate (3), the carrying slit (4), and the flowcell (5) which fits to plate 2 in an angle of 90°.*

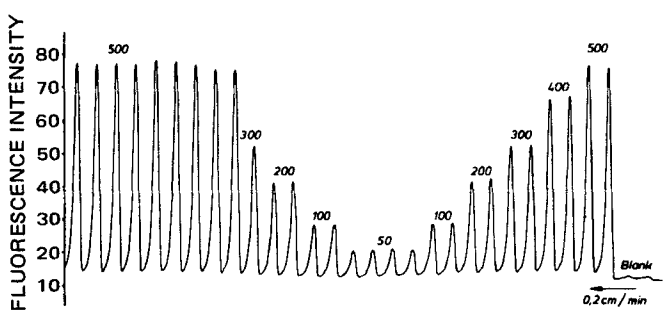


Figure 5—*Chart recordings of the determination of canrenone in serum (50–500 μg/liter).*

[3] Sampler IV and proportioning pump III of AutoAnalyzer II, Technicon GmbH, Bad Vilbel, Germany.
[4] Model 203, Perkin-Elmer GmbH, Ueberlingen, Germany.
[5] Kompensograph III, Siemens AG, Karlsruhe, Germany.
[6] Autolab Minigrator, Spectra Physics GmbH, Darmstadt, Germany.
[7] Model 9830, Hewlett-Packard, Frankfurt, Germany.
[8] Triton X-100.
[9] Acidflex, Technicon GmbH.

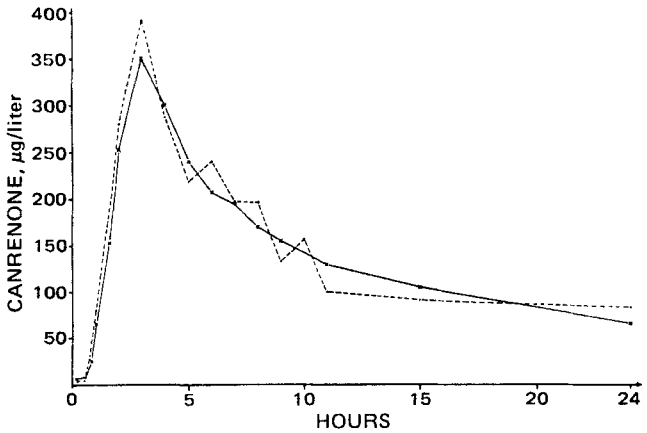


Figure 6—*Blood canrenone level after oral administration of 100 mg of spironolactone. Data determined by the manual (- - -) and automated (—) procedures are compared.*

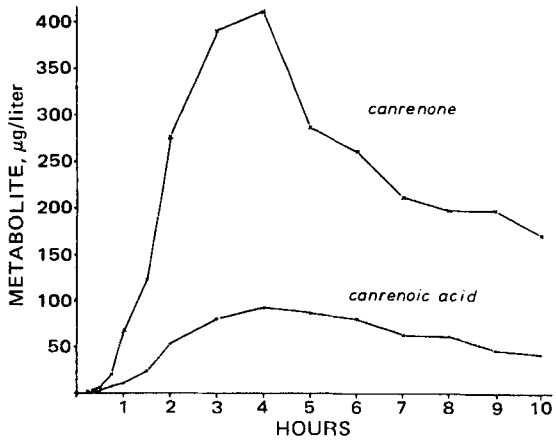


Figure 7—*Blood canrenone and canrenoic acid levels after oral administration of spironolactone determined with automated procedure.*

itated by the addition of wetting agents[8]. Carryover, a particular susceptibility of two-phase systems, was avoided by setting the number of samples relatively low, 10/hr. Furthermore, the ratio of sample volume to wash solution volume was set at 1:5. Thus, with proper functioning, the effect on the analytical results by carryover from sample to sample was very small and correction was not necessary.

A very high sensitivity of fluorescence measurement was achieved by optimally mounting the flowcell in the light path of the fluorometer. This was accomplished by having a cell holder that was adjustable in two axes (Fig. 4).

The fluorescence measured in the two fluorometers was recorded on two separate recorders. The records obtained (Fig. 5) showed that washing with water between individual samples was virtually complete so that the initial baseline was reached between sample peaks. With the aid of an integrating device[6], the peak heights were calculated from the intensity of the fluorescence and were printed out.

A good correlation existed between canrenone concentration and peak height. The curve was linear from 50 to 5000 μg of canrenone or canrenoic acid/liter. The lowest limits of detection were 20 μg/liter for canrenone and 30 μg/liter for canrenoic acid; *i.e.*, when using a 0.23-ml sample, less than 10 ng of the metabolite could be detected quantitatively.

Figure 6 shows the blood level curve for canrenone up to 24 hr after oral administration of 100 mg of spironolactone. The manually determined canrenone concentrations were compared with those obtained with the automated procedure. Control analyses of serum samples with known canrenone concentrations were included at regular intervals (*e.g.*, every 15 samples) to allow detection of time delays and to check the precision and accuracy of the analyses. From 50 analyses of control serum with 500 μg of canrenone/liter, a coefficient of variation from day to day of 5.2% was determined. Determination of canrenoic acid gave a somewhat higher value of 6.3%. From multiple analyses of one specimen with the same concentration (Fig. 5, 500 μg of canrenone/liter), a coefficient of variation in series of 1.8% was calculated.

Figure 7 shows the canrenone and canrenoic acid blood levels obtained after oral administration of 100 mg of spironolactone determined with the automated procedure.

Partition of canrenone between the aqueous and organic phases was not quantitative and reached a certain equilibrium only, in contrast to the manual procedure where phase distribution was almost complete. Consequently, a certain proportion of canrenone remained in the aqueous phase and was determined together with canrenoic acid. The value for canrenoic acid, therefore, had to be corrected for the amount of previously unextracted canrenone as follows:

$$C_{CS} = C_{C/CS} - FC_C \qquad \text{(Eq. 1)}$$

where:

C_{CS} = corrected value of canrenoic acid concentration
$C_{C/CS}$ = uncorrected value of canrenoic acid concentration containing canrenone determined as canrenoic acid with canrenoic acid standard
C_C = canrenone concentration of same sample determined with canrenone standard
$F = C_T/T$ where T is the concentration of canrenone standard, and C_T is that part of the canrenone standard T that is determined as canrenoic acid with a canrenoic acid standard

In spite of the comparatively low number of analyses of 20 determinations/hr, the time saved by automation is considerable in comparison with the manual procedure. Canrenone and canrenoic acid analyses to determine the blood level curves, which previously took 2 days, can now be carried out in 2–3 hr. The sensitivity was adequate in spite of the one-third lower serum requirement, and the precision was considerably increased.

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ACKNOWLEDGMENTS AND ADDRESSES

Received April 2, 1976, from the *Department "Bioanalytik" of Chemical and Pharmaceutical Research, Boehringer Mannheim GmbH, 6800 Mannheim, West Germany.*

Accepted for publication September 24, 1976.

Presented in part at the Annual Meeting of the Federation of German Chemical Society, Cologne, West Germany, September 1975.

The authors thank Mr. K. E. Wallach for designing the cell holder and Mr. G. Kilian for technical assistance.

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