the Bureau of Standards," No. 303, U. S. Government Printing Office, Washington, D. C., 1917, p. 205.

(3) "Handbook of Chemistry and Physics," 47th ed., Weast, R. C., ed., The Chemical Rubber Publishing Co., Cleveland, Ohio, 1966, p. E 176.

(4) Argauer, R., and White, C., *Anal. Chem.*, **36**, 368 (1964).

(5) Woods, L., and Sapp, J., *J. Chem. Eng. Data*, **8**, 235 (1963).

(6) Riggs, J. L., Seiwald, R. J., Burckhalter, J. H., Downs, C. M., and Metcalf, T. G., *Am. J. Pathol.*, **34**, 1081(1958).

(7) Lippert, E., Nagele, W., Seibold-Blankenstein, I., Staiger, U., and Voss, W., *Z. Anal. Chem.*, **179**, 1(1959).

Keyphrases

Fluorometry—analysis
Eye response—fluorometry, comparable
Quinine—azonaphthalene sulfonic acid dye aluminum chelate, reference source
Rhodamine B—fluorescence standard

Quantitative Analysis and Alkaline Stability Studies of Allopurinol

By PHILIP D. GRESSEL and JOSEPH F. GALLELLI

Methods for quantitative chromatographic separation and spectrophotometric analysis are reported for allopurinol [4-hydroxypyrazolo(3,4-*d*) pyrimidine] in the presence of its alkaline decomposition products. Stability data are presented using rate constants obtained from Arrhenius-type plots; $t_{90\%}$ for an unbuffered sodium allopurinol solution is approximately 150 days at 25°. The formate salt of 3-amino-4-pyrazolecarboxamide accounts for essentially all the degradation under pharmaceutically usable conditions.

ALLOPURINOL, 4-hydroxypyrazolo(3,4-*d*)pyrimidine, is a new, potent inhibitor of xanthine oxidase used in the treatment of hyperuricemia associated with gout. Extensive studies with the parenteral form of this drug have been performed by the National Cancer Institute to evaluate its effectiveness in the prevention of hyperuricemia and uric acid nephropathy in patients treated with X-ray and/or chemotherapy. The chemical stability studies in the present work were oriented toward conditions likely to be encountered in the clinical studies.

Some preliminary work (1) had been done previously on the assay of allopurinol solutions, using the ratio of optical absorbance at 262 mμ:255 mμ. It is found, however, that the UV spectrum of the major decomposition product does not differ enough from that of allopurinol, at any pH, to enable one to perform accurate determinations (see Fig. 1). The preliminary work (1) utilizing simple spectrophotometric analysis had not resulted in the detection of any decomposition of a refluxing solution of sodium allopurinol. However, thin-layer chromatography conducted in this laboratory on similar samples clearly showed the presence of two compounds in significant amounts. This led to the search for a larger scale quantitative method of separation of the components in aqueous solutions.

A specific method of assay was developed for allopurinol in the presence of its decomposition products, utilizing ion-exchange chromatographic separation and spectrophotometric analysis. Kinetic studies employing the assay method were done for some alkaline conditions.

Received June 30, 1967, from the Pharmaceutical Development Service, Pharmacy Department, Clinical Center, National Institutes of Health, Bethesda, MD 20014
Accepted for publication September 19, 1967.
The authors express their appreciation to Miss Gertrude B. Elion and Dr. George H. Hitchings, Burroughs Wellcome & Co., Tuckahoe, N. Y., for their assistance in the identification of the degradation product and for supplying authentic samples. They are also indebted to Dr. Henry Fales, National Heart Institute National Institutes of Health, for performing and interpreting NMR and mass spectra.

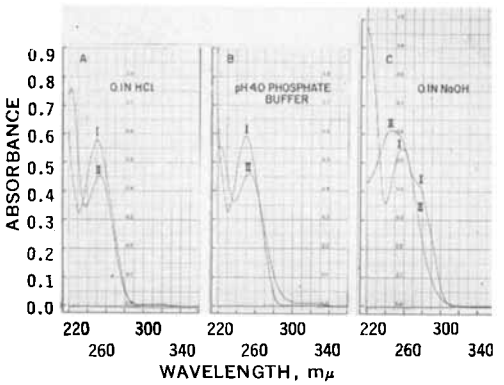


Fig. 1—Ultraviolet spectra of allopurinol (I) *and 3-amino-4-pyrazolecarboxamide* (II), *1.04 × 10⁻² M, in 0.1 N HCl, pH 4.0 phosphate buffer (0.1 M phosphate, 0.2 M NaCl), and 0.1 N NaOH.*

EXPERIMENTAL

Reagents—Allopurinol,[1] m.p. greater than 320°, was confirmed by IR spectrum (no official standards exist).

A strongly basic ion-exchange resin,[2] 200–400 mesh, was used; it was washed alternately with large volumes of 2 *N* HCl, water, and 2 *N* NaOH, (2) until the effluent was clear both by visual inspection and by ultraviolet spectrum. Then the procedure was repeated with the phosphate buffers, visual inspection. The pH 10 washings were continued until there was no change in pH. Stock solutions of phosphate buffers were prepared as follows: (*a*) pH 10 buffer, 0.100 *M* Na_2HPO_4, 0.200 *M* NaCl, adjusted to pH 10.00 with NaOH; (*b*) pH 4 buffer, 0.100 *M* $NaH_2PO_4 \cdot H_2O$, 0.200 *M* NaCl, adjusted to pH 4.00 with HCl.

Silica gel containing a fluorescent material[3] was

[1] Obtained from Burroughs Wellcome & Co., Tuckahoe, N. Y.

[2] Marketed as Dowex-1X8 (chloride form) by the Dow Chemical Co., Midland, Mich.

[3] Silica gel GF_{254} according to Stahl for thin-layer chromatography, manufactured by E. Merck Ag., Darmstadt, Germany; distributed by Brinkmann Instruments Inc., Westbury, L. I., N. Y.

TABLE I—THIN-LAYER CHROMATOGRAPHIC SEPARATION OF ALLOPURINOL FROM ITS DEGRADATION PRODUCTS

Solute	Eluent	R_f
Allopurinol	Chloroform–USP ethanol, 3:2	0.64
3-Amino-4-pyrazolecarboxamide	Chloroform–USP ethanol, 3:2	0.47
Degradation product of allopurinol	Chloroform–USP ethanol, 3:2	0.47
3-Amino-4-pyrazolecarboxamide hemisulfate hydrate	Chloroform–USP ethanol, 3:2	0.47
Degradation product of allopurinol from H_2SO_4 soln.	Chloroform–USP ethanol, 3:1	0.35
Partially degraded allopurinol soln.	Chloroform–USP ethanol, 3:1	0.14, 0.35
Partially degraded allopurinol soln.	Acetone, A.R.	0.33, 0.57
Allopurinol	Acetone, A.R.	0.57

Ion-Exchange Chromatographic Separation Procedure—Columns were packed with slurries of the anion-exchange resin in pH 10 phosphate buffer. Methods of packing and eluting the column and detecting the solutes in the effluent are essentially the same as those used in previous work conducted in this laboratory (3). The pH 10 buffer was used to elute the pure allopurinol fraction; then pH 4 buffer was substituted to elute the decomposition product from the columns. Effluent fractions corresponding to individual peaks were pooled and quantitatively diluted in pH 4 phosphate buffer to obtain ultraviolet spectra for quantitative analysis.

Columns were regenerated for reuse by pumping 300 ml. of the pH 4 phosphate buffer, then 300 ml. of the pH 10 phosphate buffer, through the apparatus.

Kinetic Studies—Chemical assays using the separation techniques above were performed on aliquots of solutions kept under various conditions (see Table II) at time intervals varying with observed reaction rates. Studies at the listed conditions were conducted at 37° in a constant-temperature water bath, and at 52° in a constant-temperature oven. One study was done at those temperatures as well as at 76° in a constant-temperature oven.

TABLE II—STABILITY STUDIES: CONDITIONS AND RESULTS
(k = RATE CONSTANTS IN RECIPROCAL HOURS)

Soln.	Concn. mg./ml.	pH at 25°	Solvent	Observed $k_{76°}$	Observed $k_{52°}$	Observed $k_{37°}$	Extrap. $k_{25°}$	Calcd. $t_{90\%}$ 25°, Days
A	5	10.70	0.2 *M* PO_4^- buffer	...	2.57×10^{-3}	5.68×10^{-4}	1.54×10^{-4}	28.5
B	5	11.50	0.2 *M* PO_4^- buffer	...	3.85×10^{-3}	7.48×10^{-4}	1.84×10^{-4}	23.9
C	5	10.80	NaOH, *q.s.*	1.22×10^{-2}	1.09×10^{-3}	1.38×10^{-4}	2.92×10^{-5}	150
D	5	11.50	NaOH, *q.s.*	1.74×10^{-2}	1.96×10^{-3}	...	1.03×10^{-4}	42.6
E	1	10.80	*A* × 20/100 in 0.9% NaCl	...	1.57×10^{-3}	3.19×10^{-4}	7.82×10^{-5}	56.3
F	1	11.50	*B* × 20/100 in 0.9% NaCl	...	2.20×10^{-3}	5.36×10^{-4}	1.82×10^{-4}	24.3
G	1	10.50	*C* × 20/100 in 0.9% NaCl	...	4.32×10^{-4}	1.13×10^{-4}	4.00×10^{-5}	110

used in the thin-layer chromatographic procedures.

All other chemicals were reagent grade, except as specified.

Apparatus—Thin-layer chromatography plates were prepared with a Desaga thin-layer chromatography kit.[4] Chromatographic column elution under positive pressure was performed with a Sigmamotor pump.[5] The eluent was monitored with a GME ultraviolet absorption meter, model UV 265 IF;[6] percent transmittance was recorded on a Texas Rectilinear recording milliameter.[7] Borosilicate glass columns, 1 cm. inner diameter, 25 cm. in length, and fitted with ground-glass connections for a reservoir for packing or eluting under air pressure, were used to hold the ion-exchange resin. Ultraviolet analyses were carried out on either a Beckman model DU spectrophotometer or a Cary 11 recording spectrophotometer. Infrared spectra were obtained on a Beckman IR5A spectrophotometer. Melting points were obtained on a Thomas-Hoover melting point apparatus.[8] Constant-temperature ovens (±1°) and a constant-temperature water bath (±.02°) were used in stability studies.

Thin-Layer Chromatographic Separation Procedure—Activated layers 250 μ thick, of Silica Gel GF_{254}, were used for qualitative studies. Spots were detected by their quenching of fluorescence under ultraviolet light. (See Table 1).

Determination and Identification of the End Product of Degradation—Recovery of the major end product was accomplished by two methods. In one procedure, solutions in the phosphate eluents were vacuum distilled to dryness; the residue was extracted with *n*-butanol to isolate the free base. In the second procedure, solutions were made acidic (pH 1–2) with sulfuric acid, concentrated to about one-half their original volume by vacuum distillation, then refrigerated for several days to obtain crystals of the sulfate salts. Ultraviolet, infrared, NMR, and mass spectral analyses were performed, as well as elemental analyses. Retention of the unknown on a cation-exchange resin[9] was observed. Duclaux values (4) were obtained on a dilute solution of distillate from a sample of allopurinol solution which had been refluxed for 15 hr. in sufficient excess NaOH to make the solution pH 11.7 at 25°. Melting point data were obtained on the sulfate salt of the unknown, known 3-amino-4-pyrazolecarboxamide hemisulfate hydrate, and on an intimate mixture of the known and unknown.

Results and Discussion

End Products of Reaction—All the evidence points to 3-amino-4-pyrazolecarboxamide as the

[4] Distributed by Brinkmann Instruments, Inc., Westbury, L. I., N. Y.

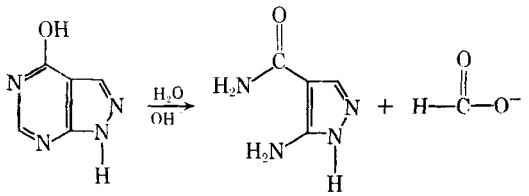
[5] Sigmamotor, Inc., Middleport, N. Y.

[6] Gilson Medical Electronics, Middleton, Wis.

[7] Texas Instruments, Inc., Houston, Tex.

[8] Arthur H. Thomas Co., Philadelphia, Pa.

[9] Dowex-50.



Scheme I

only significant end product. The overall reaction is shown in Scheme I.

Spectral evidence further confirms 3-amino-4-pyrazolecarboxamide; the IR spectra of known and unknown 3-amino-4-pyrazolecarboxamide are identical in either the free basic form or the salt form, although the spectra of the free form of either differ significantly from those of the salt form. UV spectra are identical throughout the normal pH scale. High resolution mass spectra of the degradation product show a parent peak with mass 126.0526. Calculated formula weight for 3-amino-4-pyrazolecarboxamide is 126.0541. The melting point of an admixture of its known and unknown hemisulfate hydrate is not depressed: known, 220–221°; unknown, 219–220°; equal mixture, 221–222°, all uncorrected.

The reaction scheme was confirmed indirectly by the confirmation of formate in a rapidly degraded solution. Duclaux values (3) obtained on the distillate were 2.7%, 2.9%, and 3.1%, respectively. The values for a correspondingly low concentration (0.2%) of authentic formic acid were 2.8%, 3.3%, and 3.6%.

Ion-Exchange Recovery of Allopurinol—The acidic nature of allopurinol, as well as the basic nature of its degradation product, coupled with their solubility in very polar solvents, made allopurinol a suitable candidate for ion-exchange chromatographic separation. In this work allopurinol was quantitatively retained on the column as the anion while 3-amino-4-pyrazolecarboxamide was being eluted off along with nonionic constituents. (See Fig. 2.) Then the pure allopurinol fraction was quantitatively removed in the pH 4 elution.

The pKa of allopurinol is approximately 9.4. Thus a pH of at least 10 was required to have the molecule substantially in its anionic form. The choice of anion-exchange resins, therefore, was limited to those which have strongly basic groups whose ionization was not materially affected at the required pH's.

Calculation for Intact Allopurinol—Allopurinol follows Beer's law in absorbance range 0–2 under controlled conditions. At pH 4, using the phosphate eluent, $\lambda_{max.} = 250$ mμ. Molar absorptivity (ϵ) = $7.74 \times 10^3/M$; absorptivity (a) = 56.5. No interference is encountered from nonionic compounds, which have been eluted in the first fraction.

Stability of Allopurinol Solutions—Results of the kinetic studies are found in Table II. In each case there was reasonably good adherence to first-order kinetics with respect to allopurinol. (See Fig. 3.) The trials at different pH's under otherwise similar conditions indicate a first-order dependency on hydroxyl ion concentration. Arrhenius-type plots were used to extrapolate higher temperature data to 25°. (See Fig. 4.) Activation energy of the reactions was about 25,500 cal./M.

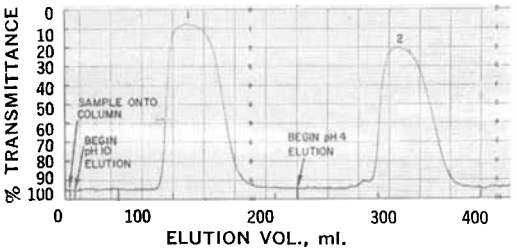


Fig. 2—Typical ion-exchange column chromatogram of a partially degraded sample of allopurinol.

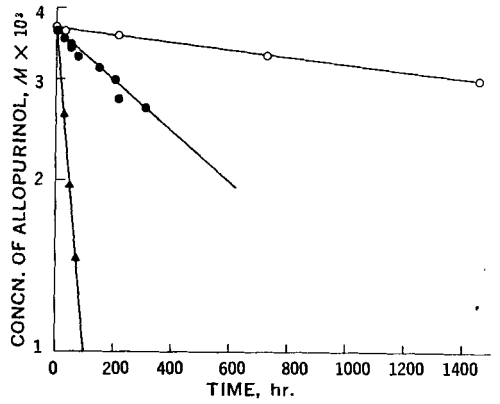


Fig. 3—Typical first-order plots showing the disappearance of allopurinol as a function of time at 37°(○), 52° (●), and 76° (▲), under otherwise similar conditions (solution C, Table II).

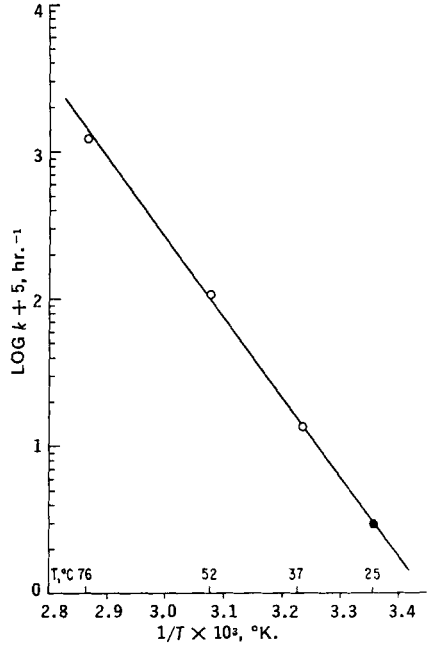


Fig. 4—Arrhenius-type plot of a typical stability study (solution C, Table II).

CONCLUSIONS

A quantitative method of assay has been found for allopurinol in the presence of inorganic salts, carbohydrate fillers, and its only significant alkaline degradation product. The method utilizes anion-exchange chromatography followed by spectrophotometric analysis.

The major product of alkaline degradation of allopurinol under pharmaceutically usable conditions is 3-amino-4-pyrazolecarboxamide, as the formate salt.

The lowest pH commensurate with the desired solubility should be used in formulating injections. At pH 10.8, an unbuffered solution of allopurinol has a $t_{90\%}$ of about 150 days.

REFERENCES

(1) Hitchings, G. H., private communication.
(2) Morris, C. J. O. R., and Morris, P., "Separation Methods in Biochemistry," Interscience Publishers, Inc., New York, N. Y., 1964, pp. 290–291.
(3) Gallelli, J. F., and Yokoyama, G., *J. Pharm. Sci.*, 56, 387(1967).
(4) McElvain, S. M., "The Characterization of Organic Compounds," rev. ed., The Macmillan Co., New York, N. Y. 1953, pp. 107, 140.

Keyphrases

Allopurinol–assay
Stability—alkaline solution
Degradation products—identity
Column chromatography—separation
TLC—separation
UV—structure, analysis
IR—structure
NMR
Mass spectroscopy

Investigation of the Use of Derivative Neutron Activation Analysis for Drug Assay

By W. A. SKINNER, M. A. LEAFFER, and R. M. PARKHURST

The use of neutron activation analysis for assay of chlorine- or bromine-containing drugs or derivatives of drugs was investigated. The ubiquitous nature of chloride greatly limited the use of this analytical method for chlorine-containing derivatives because of the background problem. Bromine-containing derivatives of aspirin and salicylic acid were prepared for neutron activation analysis, but again background bromide and interfering substances limited detection to 1×10^{-2} mcg. of bromide. Sensitivity of this analytical method is not comparable to that attainable with gas chromatography or spectrofluorometry.

In 1964, Steim and Benson (1) studied the use of derivative activation chromatography as a method for analysis of bromine-containing derivatives of amino acids, carboxylic acids, keto acids, sugars, and unsaturated fatty acids. The lower limits for detection of most amino acids separated as *N*-brosyl derivatives on paper were 0.01 mcg., as were the limits for citric or lactic acid as their bromophenacyl esters. The major limitation in sensitivity was the background arising from activation of contaminants in the paper. Conventional G-M counting was used.

There has been interest in sensitive methods for the assay of drugs in plasma or urine and it was decided to investigate the use of derivative neutron activation analysis for this purpose.

EXPERIMENTAL

Activation Source and Counting Methods—The Triga Mark I nuclear reactor at General Atomics, San Diego, Calif., with a thermal neutron flux of 1.8×10^{12} n/cm.^{2}-sec. was used for these studies. In the case of chloride determinations, the 1.64 Mev. gamma ray of 37.3 min. ^{38}Cl was used for identification and quantitative measurements while with bromide, the 0.78 Mev. gamma ray of 35.7 hr. ^{82}Br was used. Measurements were by the use of multichannel gamma ray spectrometry. Counting for bromide samples was on the fourth day after irradiation to allow short-lived isotopes to decay. Activation time in the reactor was 30 min.

Assay of Chlorine-Containing Drugs—In order to investigate the problems of background related to chloride analyses, chlorpromazine was used as a model drug. The paper selected for paper chromatographic and neutron activation studies was Schleicher and Schuell No. 589 which reportedly (1) has a lower background than others. The solvent system used was *n*-butanol–acetic acid–water (60:15:25). Chlorpromazine hydrochloride was found to move near the solvent front (R_f, 0.93) in this system. The high R_f has the advantage that any oxidation products of the drug are more polar and will trail the drug on the paper. Detection of 1 mcg. of chlorpromazine hydrochloride on Silica Gel G thin-layer plates could be made with ultraviolet light, ceric sulfate spray, or 50% sulfuric acid spray.

The chloride background of SS No. 589 white C paper was investigated prior to initiating studies with the drug. Neutron activation was for 30 min. at a flux of 1.8×10^{12} n/cm.^{2}-sec. Wide variation in chloride content of various sections of the paper

Received July 6, 1967, from the Department of Pharmaceutical Chemistry, Stanford Research Institute, Menlo Park, CA 94025
Accepted for publication September 20, 1967.
This work was supported by contract PH 43-65-1061 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.