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.

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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

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TABLE I—ANALYSIS OF BROMIDE IN THE *p*-BROMOPHENACYL DERIVATIVE OF ASPIRIN BY NEUTRON ACTIVATION ANALYSIS AFTER CHROMATOGRAPHY

Section (cm. from Bottom)	Wt. of Silica Gel G, mg.	mcg. Br (Found)
4–6	33.7	ND[a] < 0.071
6–8	32.8	ND < 0.067
8–10	43.4	8.88 ± 0.15
10–12	35.0	0.238 ± 0.032
12–14	49.2	ND < 0.085

[a] Not detected.

TABLE II—ANALYSIS OF BROMIDE IN THE *p*-BROMOPHENACYL DERIVATIVE OF ASPIRIN BY NEUTRON ACTIVATION ANALYSIS

Wt. of Silica Gel G, mg.	mcg. Br (Found)	mcg. Br. (Theoret.)
26.0	0.947 ± 0.034	1.00
34.3	ND ± 0.072	0.10
41.7	ND ± 0.071	Blank

was noted. Two-by-two inch strips from bottom to top had chloride contents of 20.4 ± 0.5 mcg., 21.0 ± 0.7 mcg., 37.0 ± 0.7 mcg., and 41.7 ± 0.7 mcg. Washing the paper with deionized, distilled water for 8 hr. reduced the background of the 2 × 2-in. strips to 5 mcg. Other papers besides SS No. 589 white C paper were investigated for their chloride content and best results were obtained with SS 589 orange paper. Washing the paper for 3 days with 10% acetic acid followed by 7 additional days of washing with deionized, distilled water resulted in chloride values of 0.5 to 2.7 mcg. for 0.5 × 1.0-in. sections of paper.

Various silica gels for TLC were analyzed for chloride content by neutron activation. Values ranged from 0.3 to 3.5 mcg. of chloride per 5 mg. of Silica Gel GF (typical quantity per spot). The presence of manganese in Silica Gel H greatly decreased the sensitivity of the chloride assay.

Assay of Bromine-Containing Derivatives of Drugs—In contrast to the case of chloride, the bromide background of the paper was lower and more uniformly distributed. The average bromide background on SS No. 589 white C paper was 0.10 ± 0.02 mcg. per 2 × 2-in. section. Washing the paper for 8 hr. with deionized, distilled water reduced the background of the 2 × 2-in. sections to nondetectable levels, *i.e.*, <0.03 mcg.

Silica gels for TLC were analyzed by neutron activation analysis for bromide background. A 5-mg. sample of Silica Gel GF had nondetectable levels of bromide present.

In order to ascertain the problem of chromatographic separation and spreading on the plates, bromine-containing derivatives of aspirin and its metabolite were prepared. Reaction of acetylsalicylic acid with *p*-bromophenacylbromide yielded the *p*-bromophenacyl derivative of the drug. Similarly, reaction of *p*-bromophenacylbromide with salicylic acid yielded the corresponding derivative of a metabolite of the drug. The *p*-bromophenacyl derivative of aspirin has not been previously reported in the literature and was thus analyzed, m.p. 133–135°, uncorrected (capillary tube).

Anal.—Calcd. for $C_{17}H_{13}BrO_5$: C, 54.13; H, 3.45. Found: C, 54.25; H, 3.59.

The aspirin derivative was spotted on a plate in an amount so that 10 mcg. of bromide was at the origin. The chromatogram was developed in chloroform, the plate sectioned, and sent for neutron activation analysis of bromide. The results are shown in Table I.

Samples of the bromine-containing derivative of aspirin were spotted on a Silica Gel G plate which was not developed, but the silica gel was scraped off and analyzed for bromide. Table II shows these results.

Manganese in the silica gel interfered with the detection and reduced the sensitivity. It was found that bromine-containing derivatives could be removed from the silica gel plates with analytical grade acetone and the acetone evaporated in the polyethylene vials used for the neutron activation thus eliminating the interfering manganese. However, the polyethylene vial blank analyzed for 0.0435 ± 0.0087 mcg. of bromide due to interfering substances present in the polyethylene. A tube in which analytical grade acetone was evaporated analyzed for 0.0303 ± 0.0101 mcg. of bromide indicating that the solvent does not contribute to the background. The experience of General Atomics has been that polyethylene is the best available substance for these studies.

An empty polyethylene vial used for sample activation by General Atomics was activated and after removal from the reactor, the vial was washed with analytical grade acetone, and the washings transferred to a nonirradiated vial, evaporated to dryness, and counted for gamma rays. Less than 1.3×10^{-2} mcg. equivalent of bromide was detected. Maximum upper limits were established on the basis of 3 σ of the count rate in the area of the 0.777 Mev. photopeak of 35.8 hr. ^{82}Br. To determine whether any extractable bromide was present in Silica Gel HF, two samples were extracted with acetone and treated as above. The results were $<8.8 \times 10^{-3}$ mcg. bromide and $<1.22 \times 10^{-2}$ mcg. of bromide indicating that extraction of bromide from the silica gel did not contribute to the background.

SUMMARY

Background problems of paper or silica gel used for chromatography greatly limit the use of neutron activation analysis for chlorine-containing drugs. Washing of the paper lowered the background to a level of 0.5 to 2.7 mcg. for 0.5 × 1.0-in. sections of paper while Silica Gel GF values ranged from 0.3 to 3.5 mcg. per 5 mg. of gel.

Although background problems encountered in the neutron activation analysis of bromide in bromine-containing derivatives of drugs were much less than in the case of chloride, they were quite limiting. Silica gel was preferred to the use of paper for separation of the drug derivatives studied. Due to interference of substances present in the polyethylene vials used for sample holding during activation, it was necessary to extract the sample after activation and count in another vial after

solvent removal. With these precautions, limits of detection of bromide were approximately 1×10^{-2} mcg. which is far less sensitive than spectrofluorometric and gas chromatographic methods for certain drugs. With longer activation times perhaps the sensitivity could be increased but costs of analyses would also increase and, perhaps, background problems.

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Keyphrases

Neutron activation, derivative—analysis
Chlorpromazine—test compound
Aspirin, bromine derivatives—test compounds
Paper chromatography—separation, degradation products
TLC—separation, degradation products

Effects of Some Adrenergic Agents on Low Frequency Electroshock Seizures

By PENG NAM YEOH and HAROLD H. WOLF

The effect of several adrenergic agents on low frequency electroshock seizures was studied in normal, reserpine and α-mmT pretreated mice. Seizure threshold was elevated by pronethalol and propranolol and reduced by phenoxybenzamine and isoproterenol. Both reserpine and α-mmT antagonized the threshold altering activity of pronethalol and phenoxybenzamine. Reserpine plus pronethalol or propranolol completely abolished the central stimulation induced by isoproterenol. A central adrenergic system involving antagonistic types of receptors appears to be involved.

It has been shown that β-adrenergic blocking agents possess anticonvulsant activity when evaluated by maximal electroshock and chemoshock techniques (1, 2). Preliminary studies in these laboratories (unpublished) indicate that the β-adrenergic blocking agents pronethalol and propranolol also reduce seizure susceptibility in audiogenic seizure susceptible mice, whereas phenoxybenzamine, an α-adrenergic blocking agent, enhances this phenomenon.

It was the objective of this study to determine whether such drug effects reflect general changes in the level of brain excitability which could be readily quantitated by measuring alterations in low frequency electroshock seizure threshold (l.f. EST). Increasing evidence indicates that endogenous brain amines may play an important role in the expression of seizure states (3–6). The experiments were designed to facilitate an evaluation of the relationship between the adrenergic drug activity described and the existing level of endogenous brain amines.

EXPERIMENTAL

Adult, male, albino mice of a random bred Swiss strain (Maxfield Animal Supply, Cincinnati, Ohio) ranging from 16 to 28 Gm. were used as experimental animals. They were housed in groups of 9 to 11 animals in plastic cages (26 × 15 × 12 cm.) with wire mesh or perforated metal tops. Except during periods of experimentation, they were maintained on Purina laboratory chow and given free access to tap water.

Pronethalol [(2-isopropylamino)-1-(2-naphthyl) ethanol hydrochloride] and propranolol [(1-isopropylamino)-3-(1-naphthyloxy)-2-propanol hydrochloride] were employed as β-adrenergic blocking agents and phenoxybenzamine hydrochloride as an α-adrenergic blocking agent. The catecholamine depleting agents used were reserpine (2 mg./Kg., 24-hr. pretreatment) and α-methyl-metatyrosine (α-mmT; 400 mg./Kg., 24-hr. pretreatment).

All drugs were given intraperitoneally as solutions in distilled water except pronethalol which was administered in 0.2% methylcellulose.[1] They were prepared so that 10 ml./Kg. contained the required dose. Requisite volumes of saline or methylcellulose (0.2%) were administered to control animals. In studies where two or more drugs were employed in sequence, the control animals received only the drug (or drugs) used for pretreatment. Except for fluorometric assays, all experiments were conducted between 4 and 11 p.m. at 23–25°.

Neurotoxicity Studies—The method of Weaver and Miya (7) with slight modifications was employed to obtain the time of peak effect and the dose of each of the adrenergic agents which produced overt evidence of neurological toxicity in 50% of mice (TD_{50}). Mice were placed on a rolling bar, 2.5 cm. in diameter, rotating at 6 r.p.m. An animal was considered to be unaffected if it could stay on the bar continuously for 1 min. in any one of three consecutive trials. Data obtained in this test were statistically analyzed by the method of Litchfield and Wilcoxon (8). In all further experiments (unless otherwise stated) the dose of all adrenergic agents used was $^1/_2$ TD_{50}.

Low Frequency Electroshock Seizure Threshold (l.f. EST) Test—A Grass stimulator (model S4G, Grass Instrument Co., Quincy, Mass.) was used to apply a unidirectional current (6 pulses per second, 0.2 msec. duration, 3 sec. stimulus duration) through corneal electrodes (9). Mice were shocked at various intensities according to the staircase method of Finney (10). The presence of a "stunning" response (at least 3 sec. of immobility) or minimal clonus was con-

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[1] Methocel. Dow Chemical Co., Midland, Mich.