temperature is calculated from the slope as described previously (23). The activation energy for the transport process for each solvent system is obtained from the slope of an Arrhenius plot of log permeability constant *versus* the reciprocal of the absolute temperature (Fig. 7). The permeability coefficients at each temperature and the activation energy for each system are shown in Table IV, with the expected inverse order between the rate ratio and the activation energy being observed.

REFERENCES

(1) T. Higuchi, *J. Soc. Cosmet. Chem.*, **11**, 85 (1960).

(2) J. E. Treherne, *J. Physiol.*, **133**, 171 (1956).

(3) W. B. Shelly and F. M. Melton, *J. Invest. Dermatol.*, **13**, 61 (1949).

(4) J. B. Shelmire, Jr., *Arch. Dermatol.*, **82**, 24 (1960).

(5) E. P. Laug, E. A. Vos, F. M. Kunze, and E. J. Umberger, *J. Pharmacol. Exp. Ther.*, **89**, 52 (1947).

(6) H. Leslie-Roberts, *Br. J. Dermatol.*, **40**, 325 (1928).

(7) D. E. Wurster and S. F. Kramer, *J. Pharm. Sci.*, **50**, 288 (1961).

(8) A. Horita and L. J. Weber, *Life Sci.*, **3**, 1389 (1964).

(9) S. W. Jacob, M. Bischel, and R. J. Herschler, *Curr. Ther. Res.*, **6**, 193 (1964).

(10) R. B. Stoughton and W. Fritsch, *Arch. Dermatol.*, **90**, 512 (1964).

(11) A. M. Kligman, *J. Am. Med. Assoc.*, **193**, 796 (1965).

(12) R. J. Feldmann and H. I. Maibach, *Arch. Dermatol.*, **94**, 649 (1966).

(13) H. L. McDermot, G. W. Murray, and R. M. Heggie, *Can. J. Physiol. Pharmacol.*, **43**, 845 (1965).

(14) J. M. Stelzer, J. L. Colaizzi, and P. J. Wurdock, *J. Pharm. Sci.*, **57**, 1732 (1968).

(15) H. L. McDermot, A. J. Finkbeiner, W. J. Willis, and R. M. Heggie, *Can. J. Physiol. Pharmacol.*, **45**, 299 (1967).

(16) J. E. Wahlberg and E. Skog, *Acta Dermatol.*, **47**, 209 (1967).

(17) D. D. Munro and R. B. Stoughton, *Arch. Dermatol.*, **92**, 585 (1965).

(18) W.-W. Shen, A. G. Danti, and F. N. Bruscato, *J. Pharm. Sci.*, **65**, 1780 (1976).

(19) J. P. Astley and M. Levine, *ibid.*, **65**, 210 (1976).

(20) F. Marcus, J. L. Colaizzi, and H. Barry, *ibid.*, **59**, 1616 (1970).

(21) R. J. Scheuplein, Final Report (1965) to U.S. Army Chemical Research and Development Laboratories, Md., Contact DA 18-108-AMC-148 (A).

(22) B. Idson, *J. Pharm. Sci.*, **64**, 901 (1975).

(23) D. E. Wurster, J. A. Ostrenga, and L. E. Matheson, Jr., *ibid.*, **68**, 1406 (1979).

(24) B. Gehauf, J. Epstein, G. B. Wilson, B. Witten, S. Sass, V. E. Bauer, and W. H. C. Rueggeberg, *Anal. Chem.*, **29**, 278 (1957).

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High-Performance Liquid Chromatographic Determination of Azosemide in Commercial Animal Feed

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Abstract □ A high-performance liquid chromatographic (HPLC) method was developed for the assay of 2-chloro-5-(1*H*-tetrazol-5-yl)-4-[(2-thienylmethyl)amino]benzenesulfonamide (I) in animal feed diet mixtures. The sample is extracted with acetone, an aliquot of the extract is evaporated to dryness, and the residue is dissolved in chloroform. This solution can be subjected to either of two cleanup procedures. The first traps I on a sodium hydroxide–diatomaceous earth column. Compound I is then recovered using an acetic acid–ethyl acetate–chloroform eluent, which is removed by evaporation, followed by dissolution of the residue in aqueous sodium hydroxide. The second procedure involves the direct extraction of I from the chloroform solution. A linear relationship exists between the HPLC UV detector response at 254 nm and concentration up to 170 μg/ml. The sodium hydroxide extraction procedure resulted in an overall recovery (±*SD*) of 92.9 ± 1.6 and 97.4 ± 1.8% ($n = 5$) at levels of 200 and 6000 ppm, respectively. Overall recoveries (±*SD*) obtained by the diatomaceous earth column procedure for the 200- and 6000-ppm levels were 90.0 ± 2.0 and 95.9 ± 1.6% ($n = 5$), respectively.

Keyphrases □ Azosemide—analysis, high-performance liquid chromatography, commercial animal feed □ Diuretic agents—azosemide, high-performance liquid chromatographic analysis, commercial animal feed □ High-performance liquid chromatography—analysis, azosemide, commercial animal feed

Azosemide, 2-chloro-5-(1*H*-tetrazol-5-yl)-4-[(2-thienylmethyl)amino]benzenesulfonamide (I), is a new diuretic and saluretic agent (1). The drug has been formulated into tablets, injectable solutions, and lyophilizates in combination with other diuretics for the treatment of hydropic conditions (2, 3).

In long-term toxicological studies, drug administration to laboratory animals is usually done by incorporation into feed because of the inconvenience of individual manual dosing and the time involved in preparing individual dosages. Analytical methodology is required to determine that appropriate dosages are administered and that the drug is uniformly distributed and stable in the feed mix under the storage conditions used. Recent papers dealt with the determination of cyclobenzaprine, sulindac, halofenate, estradiol, and phenobarbital in animal feed mixtures for use in toxicity and/or carcinogenesis studies (4–6).

Reported quantitative analyses of sulfonamides in animal feed include spectrophotometric measurement of the diazotized sulfonamide after alumina column cleanup (7), GLC assay after purification by conventional extraction methods (8, 9), quantitative TLC (10), and spectrophotofluorometry and GLC after successive percolation through silica and alumina columns or successive base–acid extractions (11).

This paper presents a method for the assay of I in animal feed mix at 200- and 6000-ppm levels using high-perfor-

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Table I—Comparative Study of Cleanup Procedures with and without Soxhlet or Manual Acetone Extraction

Cleanup Procedure	Recovery of I, %: Dry Feed Mix[a] Soxhlet	Dry Feed Mix[a] Manual	Feed Extract[a]
Procedure a	—	87.1–92.2[b]	87.4–91.7[b]
Procedure b	81.4–86.0[b]	91.0–94.5[b]	94.6–15.6[c]
Successive base–acid–base	79.9–86.2[c]	91.4–93.1[b]	94.1–95.7[c]

[a] Using 0.2 mg of I/g of dry feed or 0.02 mg of I/ml of feed extract. [b] Range of three determinations. [c] Range of two determinations.

mance liquid chromatography (HPLC) after cleanup of the feed extract by two different procedures.

EXPERIMENTAL

Apparatus—The following were used: a high-performance liquid chromatograph[1] fitted with a sample injection valve with a 20-μl sample loop[2], a fixed-wavelength UV detector (254 nm), and an electronic computing integrator[3] provided with a teletypewriter[4]; a glass chromatography tube, 15 mm (i.d.) × 350 mm, with a polytef stopcock; a mechanical shaker; a centrifuge; and 50-ml centrifuge tubes fitted with polytef-lined screw caps.

Reagents and Materials—The following were used: I, mp 210° dec.[5]; diatomaceous earth[6]; animal feed[7]; 25% (v/v) acetonitrile[8] in 0.1 *M* pH 3.2 phosphate buffer; and acetic acid–ethyl acetate–chloroform (0.15:5:25). All chemicals were analytical grade.

HPLC Conditions—A 100-cm × 2.1-mm (i.d.) column packed with 37–50-μm Bondapak phenyl/Corasil[9] was used at ambient temperature. An isocratic mobile phase system of acetonitrile–0.1 *M* pH 3.2 phosphate buffer (25:75) was delivered at the rate of 1 ml/min (~600 psi). The detector was attenuated to 0.08 and 0.16 aufs for the 200- and 6000-ppm samples, respectively.

Standard Solution Preparation—About 25 mg of I was weighed accurately into a 100-ml volumetric flask. About 80 ml of 0.01 *M* NaOH was added and shaken for 15 min to dissolve the solid. The solution was diluted to volume with 0.01 *M* NaOH and mixed well. Twenty milliliters (for use with purification Procedure a) or 10.0 ml (for use with Procedure b) of this solution was pipetted into a 100-ml volumetric flask, diluted to volume with 0.01 *M* NaOH, and mixed well.

Diatomaceous Earth Column Preparation—A small glass wool plug was placed at the base of the glass chromatographic tube. Two grams of diatomaceous earth AW was transferred to the tube and tamped lightly. Three grams of diatomaceous earth and 1.5 ml of 0.1 *M* NaOH were mixed gently and transferred to the tube. The tube was tapped on a padded bench to settle the packing and then tamped very lightly but evenly.

Sample Preparation—About 10 or 5 g of sample for concentrations of 200 or 6000 ppm, respectively, was weighed accurately into a glass-stoppered, 250-ml boiling flask. About 25 ml of acetone was added, and the flask was stoppered and shaken for 20 min. The extract was filtered through a glass wool pledget into a 100-ml volumetric flask. The extraction was repeated three more times with 20-ml portions of acetone. The flask and filter were washed with acetone, and the washings were used to dilute the combined extract to volume.

About 40 ml of the solution was transferred to a centrifuge tube, and the tube was capped and centrifuged at ~2000 rpm for 2 min. Then 25.0 or 30.0 ml of the clear supernatant liquid for purification Procedure a or b, respectively, for the 200-ppm sample or 2.0 ml for both purification procedures for the 6000-ppm sample was pipetted into a glass-stoppered, 100-ml boiling flask and evaporated to dryness on the rotary evaporator at ~45°. Acetone traces were removed under a nitrogen stream, and the residue was subjected to one of the following cleanup procedures.

Procedure a: Diatomaceous Earth Column Cleanup—About 10 ml of water-saturated chloroform was added and shaken to dissolve the residue. The solution was transferred, using a Pasteur pipet, to the diatomaceous earth column, and the flow rate was adjusted to 1–1.5 ml/min. The flask was rinsed twice with 10-ml portions of water-saturated chloroform, and the rinsings were transferred to the column. The eluate was discarded.

The adsorbed I was eluted with 40 ml of acetic acid–ethyl acetate–chloroform, and the eluate was collected in a 100-ml boiling flask. The eluate was evaporated to dryness on the rotary evaporator at ~45°. Acetic acid traces were removed under a nitrogen stream. Ten milliliters of 0.01 *M* NaOH was pipetted into the flask, and the flask was stoppered and shaken vigorously for 15 min. The solution was filtered through a 5-μm filter[10].

Procedure b: Sodium Hydroxide Extraction Cleanup—The residue

Table II—Recovery of I from Spiked Feed Mix at 200 and 6000 ppm

Amount of I Added, ppm	Amount of I Found, ppm: Procedure a	Amount of I Found, ppm: Procedure b	Recovery, %: Procedure a	Recovery, %: Procedure b
		200 ppm		
199.6	174	—	87.2	—
199.3	—	188	—	94.3
200.2	185	—	92.4	—
202.0	184	190	91.1	94.1
198.2	178	186	89.8	93.8
194.7	—	177	—	90.9
201.6	—	184	—	91.3
203.7	182	—	89.3	—
Overall recovery, %			90.0	92.9
SD			2.0	1.6
		6000 ppm		
5994	—	5870	—	97.9
6034	5920	6060	98.1	100.4
5948	5570	5740	93.6	96.5
6036	5770	5800	95.6	96.1
6044	5780	5820	95.6	96.3
6030	5820	—	96.5	—
Overall recovery, %			95.9	97.4
SD			1.6	1.8

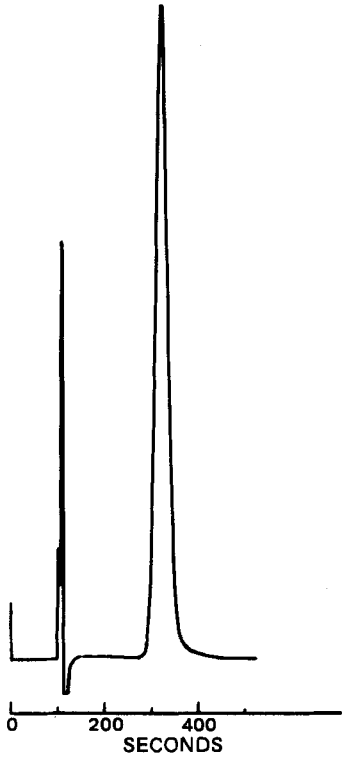

Figure 1—*Liquid chromatogram of pure I in 0.01* M *NaOH.*

[1] Model 8500, Varian Associates, Palo Alto, Calif.
[2] Model 70-10, Rheodyne, Berkeley, Calif.
[3] Model 3370 A, Hewlett-Packard, Avondale, Pa.
[4] Model ASR 33, Hewlett-Packard, Avondale, Pa.
[5] Boehringer, Mannheim G.M.b.H., West Germany.
[6] Celite 545 AW, Supelco Inc., Bellefonte, Pa.
[7] Purina Laboratory Chow, Ralston Purina Co., St. Louis, Mo.
[8] Burdick & Jackson, Muskegon, Mich.
[9] Waters Associates, Milford, Mass.
[10] Type HA, Millipore Corp., Bedford, Mass.

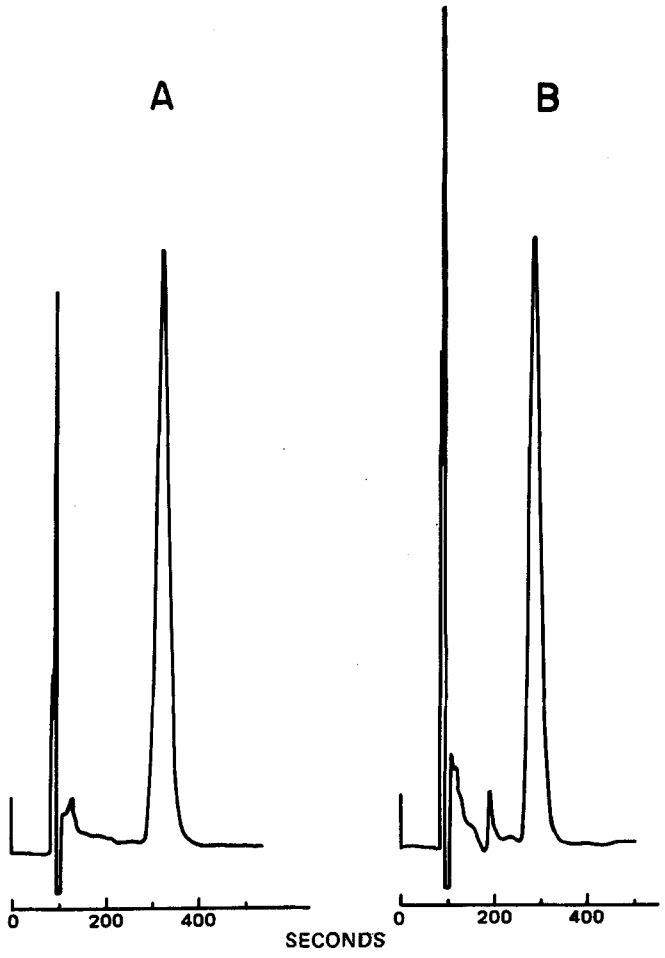

Figure 2—*Liquid chromatograms of I from 200-ppm feed sample. Key: A, Procedure a; and B, Procedure b.*

was dissolved in 10 ml of chloroform and transferred quantitatively into a centrifuge tube. The flask was rinsed twice with 10-ml portions of chloroform, and the rinsings were added to the centrifuge tube. The solution was extracted four times with 6-ml portions of 0.01 *M* NaOH. Each time, the mixture was shaken gently and centrifuged at 2000 rpm for 5 min. With a Pasteur pipet, the clear supernate was transferred carefully into a 25-ml volumetric flask. It was diluted to volume with 0.01 *M* NaOH and filtered through a 5-μm filter[10].

Chromatographic Procedure—By using the sample injection valve, 20 μl of the standard solution and 20 μl of the prepared feed sample solution were chromatographed under the described HPLC conditions. The chromatogram was quantitated by relating the peak area of I from the sample to that of I from the standard solution.

RESULTS AND DISCUSSION

Toxicological studies with I employed concentrations levels of 200 and 6000 ppm in the feed mix. Since the low level samples were expected to be more difficult to assay, the developmental work was undertaken with the 200-ppm samples. The assay of I and the cleanup procedures are based on its acidic nature.

Preliminary tests to determine the solvent for extracting I from the feed mix indicated that acetone was the solvent of choice. Attempts to extract I from the feed mix using a soxhlet extractor resulted in recoveries that were consistently lower than those obtained by manual extractions with acetone and the same sample cleanup procedure (Table I). Presumably, a fraction of I was adsorbed on the extraction thimble and/or decomposed on heating. Bowman and Rushing (6) also reported that soxhlet extractions of phenobarbital from animal feed yielded low recoveries. Soxhlet extractions were also performed with mixed solvents such as chloroform–acetone and chloroform–methanol, but the recoveries were very low.

In addition to the two sample cleanup procedures (Procedures a and b described under *Sample Preparation*), several purification procedures were attempted. A successive base–acid–base extraction was investigated whereby a methanol-containing chloroform solution of feed extract was extracted with aqueous base, followed by extraction into chloroform after acidification of the aqueous phase and reextraction into 0.01 *M* NaOH. This procedure resulted in sharp separations of the phases in each extraction, in contrast to the direct sodium hydroxide extraction cleanup procedure (Procedure b). In the latter procedure, emulsions were formed during the extraction, and the two phases could not be sharply separated even by centrifugation. Although the successive base–acid–base extraction gave cleaner liquid chromatograms than Procedure b, the recoveries using the two procedures were similar (Table I). Because of the longer analysis time required, the successive base–acid–base extraction procedure was not investigated further.

Attempts to clean up I-containing feed extracts on neutral or acid silica gel or on acid alumina columns were not successful. Trapping of I on these columns was only successful when pure I solutions were employed. In these cases, quantitative recoveries were obtained. When acetone-extractable feed constituents were present, the recoveries were always <85%. With anion-exchange columns, complete recovery of I could not be achieved even from pure solutions with various eluting solutions.

In the sodium hydroxide–diatomaceous earth column procedure (Procedure a), the sodium hydroxide strength for adsorption on diatomaceous earth and the total amount needed were determined. Various eluants and mixtures were investigated for optimum recovery of the trapped I. The addition of a bottom layer of plain diatomaceous earth in the column helped to break emulsions when they formed in the column.

Under the described HPLC conditions, I eluted as a symmetrical peak with a retention time of ~5 min (Fig. 1). The average height equivalent to a theoretical plate of the column was 1.79 ± 0.19 (*SD*) mm (n = 10). The linearity of the detector response at 254 nm was established previously. The response was linear up to 170 μg/ml.

Both cleanup procedures gave liquid chromatograms that resolved the I peak from any feed constituents present (Figs. 2 and 3). Procedure b

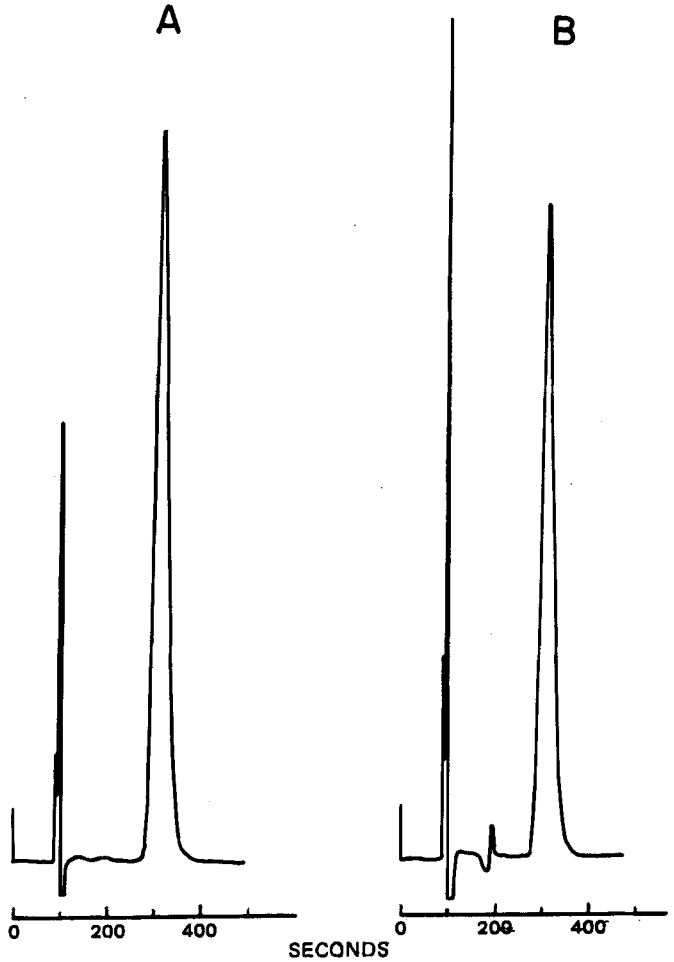

Figure 3—*Liquid chromatograms of I from 6000-ppm feed sample. Key: A, Procedure a; and B, Procedure b.*

gave a chromatogram that showed many extraneous peaks for the 200-ppm sample, but the chromatogram of the 6000-ppm sample was relatively clean. The difference on the baseline between I and the nearest extraneous peak on the 6000-ppm chromatogram was >1 min (Fig. 3). Procedure a gave very few additional peaks for the 200-ppm sample (Fig. 2). In the liquid chromatogram of the 6000-ppm sample, only two small peaks were observed.

Essentially complete recoveries were obtained when known amounts of I were analyzed in the absence of feed using both cleanup procedures. When a solution of I in acetone was added to a placebo feed, however, the overall percent recoveries ($\pm SD$) obtained by Procedure a were 90.0 ± 2.0 (n = 5) and 95.9 ± 1.6% (n = 5) for the 200- and 6000-ppm samples, respectively (Table II). Similarly, Procedure b gave overall percent recoveries ($\pm SD$) of 92.9 ± 1.6 (n = 5) and 97.4 ± 1.8% (n = 5) for the 200- and 6000-ppm samples, respectively (Table II). To provide better representation of the analytical potency, a concomitant standard may be used; *i.e.*, the standard can be subjected to the same analytical steps in the cleanup procedures.

The cleanup procedures were evaluated by spiking acetone extracts of a placebo feed with a solution of I in acetone. The recoveries, again <100%, were in close agreement with those obtained when the drug was added directly to the dry feed followed by manual extraction (Table I). Attempts to improve the recoveries by doubling the analytical sample size were not successful. Apparently, an interaction occurs between the drug and one or more of the extractable feed components, which affects the analytical recovery.

This hypothesis was supported by subsequent recovery studies using cleanup Procedure a and placebo feed samples spiked with I at the 6000-ppm level. Duplicate results of 90.9 and 92.1% were obtained when the synthetic samples were mixed using a mortar and pestle. When two similar samples were assayed 7 days after mixing, recoveries of only 82.8 and 82.5% were achieved. No peaks that could be attributed to decomposition products of I were seen in the chromatograms.

The HPLC method is stability indicating; various degradation studies showed that the I peak decreased upon decomposition with or without the appearance of new peaks.

REFERENCES

(1) A. Popelak, A. Lerch, K. Stach, E. Roesch, and K. Hardebeck, U.S. pat. 3,665,002 (Cl. 260/239.9) (1972).

(2) R. Kuhn, K. Hardebeck, H. Heinemann, and K. Osmers, U.S. pat. 3,963,834 (Cl. 424/240) (1976).

(3) R. Kuhn, K. Hardebeck, and H. Heinemann, U.S. pat. 4,031,213 (Cl. 424/229) (1977).

(4) H. B. Hucker and S. C. Stauffer, *J. Chromatogr.*, **131**, 357 (1977).

(5) M. C. Bowman and C. R. Nony, *J. Chromatogr. Sci.*, **15**, 160 (1977).

(6) M. C. Bowman and L. G. Rushing, *ibid.*, **16**, 23 (1978).

(7) C. R. Szalkowski, *J. Assoc. Off. Anal. Chem.*, **56**, 758 (1973).

(8) R. J. Daun, *ibid.*, **54**, 1277 (1971).

(9) A. Fravolini and A. Begliomini, *ibid.*, **52**, 767 (1969).

(10) U. R. Cieri, *ibid.*, **56**, 1475 (1973).

(11) J. R. King and M. C. Bowman, *Biochem. Med.*, **12**, 313 (1975).

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Potentiometric Determination of Iodine in Pharmaceutical Preparations

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Abstract □ Methods for the determination of organically bound iodine were compared. A preliminary destruction of the sample was preferable. The sample was mineralized using the Schöniger combustion. Since direct potentiometry of the iodide ion was used, further reduction of the sample was necessary. Several reductors were compared, and the best results were obtained with Devarda alloy. The proposed method was employed for the determination of iodine in X-ray contrast products. Pure compounds and pharmaceutical preparations were investigated. The coefficient of variation of the method was 0.9%.

Keyphrases □ Iodine—analysis, potentiometric, various pharmaceuticals, ion-selective electrodes □ Potentiometry—analysis, iodine in various pharmaceuticals

The increased number of commercially available ion-selective electrodes has stimulated their use in pharmaceutical analysis. The potentiometric determination of halide salts of pharmaceutical compounds such as alkaloids and phenothiazines was described (1–3). Halogen-containing organic compounds also can be determined by ion-selective electrodes. Since halide ion-specific electrodes measure only the inorganic ionic halogen in solution, preliminary liberation of the covalently bound halogen from the organic product is necessary and usually is achieved by destruction of organic matter (3–11). Methods for halogen liberation from organic compounds (hydrolysis, decomposition by acids or oxidizing acids, melting of solids, combustion in a gas stream, and oxygen flask combustion) were discussed previously (12).

Some investigators reported the liberation of organically bound iodine by a metallic reductor. Catalytic dehalogenation with sodium borohydride in the presence of palladium was described for the analysis of X-ray contrasting media (13). The use of an aluminum foil in alkaline solution was proposed for the iodine determination in liothyronine (triiodo-L-thyronine, T_3) and thyroxine (tetraiodo-L-thyronine, T_4) (14).

If a combustion method such as the Schöniger combustion (15, 16) is used to mineralize the organic matter, the halogen content can be determined by measuring the iodate or iodide content. After combustion, the iodine is present as iodine and iodate. The iodine and iodate can be reduced to iodide ions, which can be measured potentiometrically with an iodide-ion-selective electrode. In this case, a reduction step is necessary. Reduction of iodate has already been described.

Stannous chloride was used for iodate determination in iodized cooking salt with an iodide-selective electrode (17), and use of an aluminum foil in alkaline solution was reported for the iodate reduction (18). The purpose of this

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