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THIN-LAYER DETECTION OF DIAZEPAM AND/OR CHLORDIAZEPOXIDE ALONE OR IN COMBINATION WITH MAJOR DRUGS OF ABUSE IN DRUG ABUSE URINE SCREENING PROGRAMS

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SUMMARY

Three extraction procedures for the detection of diazepam, oxazepam, chlorazepate and/or chlordiazepoxide in human urines are presented. All three procedures are based on the acid hydrolysis of benzodiazepines and/or their conjugated metabolites to give the corresponding benzophenones. Procedure I involves the direct acid hydrolysis of raw urine and is recommended when the aim is to test the abuse of benzodiazepine derivatives only. Procedure II is a two-step extraction method in which a wide variety of drugs of abuse including cocaine (test based on the detection of benzoylecgonine) are extracted by the first step using paper loaded with cation-exchange resin and the benzodiazepines are tested in the second step by the acid hydrolysis of the spent urine left after removing the ion-exchange paper. Procedure III involves the use of inert fibrous matrix and then its acid hydrolysis.

The detection procedure is based on the identification of methylaminochlorobenzophenone (MACB) and aminochlorobenzophenone (ACB). MACB is detected as a yellow-colored compound while ACB is detected by spraying with Bratton–Marshall reagent. Specificity of detection of ACB has been achieved by the selection of a thin-layer developing solvent system in which sulfonamides with primary aromatic amino groups remain at the origin.

INTRODUCTION

Benzodiazepines, which are structurally and pharmacologically unrelated to the phenothiazines, are known as minor tranquilizers. The biotransformation of benzodiazepines in the human body has been discussed recently by Kaistha[1]. The methods for the detection of benzodiazepines in biological fluids have to be based on the identification of their metabolites because of their lack of availability in sufficient amounts in the free unchanged form[2].

The metabolism of chlordiazepoxide (I) in man has been investigated by Koechlin *et al.*[3] and Schwartz and co-workers[4,5]. The drug is first demethylated to N-desmethylchlordiazepoxide (II), which is further metabolized by the liver to the

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lactam, demoxepam (III) and desoxydemoxepam; all of these metabolites are reported to be pharmacologically active. Demoxepam (III), the major metabolite of chlordiazepoxide, has been shown by Schwartz and Postma[6] to be biotransformed in man to nordiazepam (VI) and oxazepam (VII). Subsequently, Dixon *et al.*[7] reported that nordiazepam was a measurable plasma metabolite of chlordiazepoxide following chronic oral administration. In a personal communication, De Silva pointed out that Brooks *et al.*[8] have been able to measure oxazepam glucuronide in the urine of a few overdose cases following ingestion of chlordiazepoxide. Oxazepam was extracted after incubation of urine with glucuronidase sulfatase.

Kimmel and Walkenstein[9] were the first to report oxazepam glucuronide as a metabolite of chlordiazepoxide in the dog. Acid hydrolysis of chlordiazepoxide and/or its major metabolites, including oxazepam glucuronide, yields 2-amino-5-chlorobenzophenone (ACB, IV), which is a yellow-colored derivative. Chlordiazepoxide and N-desmethylchlordiazepoxide can be measured in blood and plasma spectrofluorimetrically by the methods described by Koechlin and D'Arconte[10], Schwartz and Postma[4] and Strojny *et al.*[11]. A spectrophotodensitometric determination has also been reported for chlordiazepoxide by Strojny *et al.*[11]. Zingales[12] and Vandemark and Adams[13] reported a gas chromatographic (GC) procedure using electron-capture and nitrogen detectors for the determination of unchanged chlordiazepoxide in plasma and serum, respectively. Allison *et al.*[14] reported a GC procedure based on acid hydrolysis of the unchanged drug and/or its metabolites. A quantitative colorimetric procedure for the total assay of urinary metabolites is also available[2,15]. The colorimetric method is based on the acid hydrolysis of metabolites into the corresponding benzophenones, which are then coupled with the Bratton–Marshall reagent[16] for primary aromatic amines. The same reaction was applied by Kokoski *et al.*[17] for the thin-layer detection of chlordiazepoxide and/or its metabolites and other benzodiazepine derivatives in human urine.

The metabolism of diazepam (V) has been reported by Kaplan *et al.*[18], Greenblatt and Shader[19], De Silva and Puglisi[20], Schallek *et al.*[21], Blacow[22] and Schwartz *et al.*[23]. The drug is completely biotransformed in the body through demethylation, hydroxylation and conjugation processes. The major metabolites are (i) N-desmethyldiazepam or nordiazepam (VI), (ii) 3-hydroxydiazepam (temazepam, VII) and (iii) oxazepam (VIII); all of these metabolites are pharmacologically active. The major urinary metabolite is oxazepam glucuronide, with small amounts of 3-hydroxydiazepam glucuronide[19,23]. The excretion of conjugated desmethyldiazepam has been reported to be 2.5–9% of the administered dose[18]. Oxazepam and 3-hydroxydiazepam glucuronides are measured, after acid hydrolysis of urine specimens, as 2-amino-5-chlorobenzophenone (ACB, IV) and 2-methylamino-5-chlorobenzophenone (MACB, IX), respectively. The same reaction was applied by De Silva and coworkers[24,25] in their studies on blood levels of diazepam and its metabolites. Meola and Brown[26] reported a thin-layer chromatographic (TLC) procedure for the detection of diazepam employing enzymatic hydrolysis of oxazepam glucuronide. Haden *et al.*[27] reported an enzyme multiplied immunoassay procedure (EMIT) for the detection of diazepam, oxazepam and N-desmethyldiazepam.

The major metabolite of diazepam, oxazepam, is sold under the trade-name Serax. Oxazepam metabolizes rapidly in the liver and at the extra-hepatic sites to its glucuronide conjugate; only 1% of an orally administered dose is excreted in an

unchanged form[28]. It has been reported that the half-life ranges from 3 to 21 h. Another benzodiazepine drug, chlorazepate, sold under the trade-name Tranxene, is biotransformed into nordiazepam, a primary blood metabolite[29]. Oxazepam glucuronide is likely to be one of the urinary metabolites of chlorazepate also.

This paper describes three procedures for the detection of benzodiazepines in human urine. Each procedure has its advantages, depending on the needs of the clinical program and/or working capacity of a toxicology laboratory facility. Procedure I is recommended when the aim is to test for the abuse of benzodiazepine drugs only. Procedure II is a two-step extraction method in which a wide variety of drugs of abuse can be detected in addition to benzodiazepines. Procedure III uses an inert fibrous matrix[30] method and is recommended to laboratories who prefer not to deal with raw urines. All three procedures involve acid hydrolysis of benzodiazepines, their metabolites and/or their conjugates to the corresponding benzophenones.

EXPERIMENTAL

Procedure I. Single-step acid hydrolysis of raw urine for the detection of benzodiazepines

A 20–50-ml volume of undiluted urine is transferred to a 4-oz. wide-mouthed screw-capped jar, to which 5 ml of concentrated hydrochloric acid are added and swirled. The jar is then placed in an oven maintained at 100° for 1 h. The jar is cooled to room temperature and the contents are adjusted to pH 10.1 by addition of 10 ml of ammonium chloride–ammonium hydroxide buffer[31]. A 30-ml volume of chloroform–isopropanol (5:2) is added and the jar is then shaken on a reciprocating shaker for 20 min. The lower organic phase is pipetted into a 50-ml non-graduated conical centrifuge tube containing 5 drops of 0.5% sulfuric acid in methanol. The extract is evaporated in an oven having a horizontal air flow and maintained at 85–90°. The residue along the sides of the tube is washed with about 0.5 ml of methanol, vortexed and the sides of the tube are again washed with a further few drops of methanol. The methanol is evaporated to dryness as above. The residue is spotted on a chromatographic plate as described under *Detection Procedure A*, for benzodiazepines.

Procedure II. Two-step extraction of poly-drugs, benzoylecgonine and benzodiazepines

(a) Opiates, amphetamines, barbiturates and miscellaneous drugs of abuse (poly-drugs). A wide variety of drugs of abuse, as reported earlier[32–36], are absorbed on a 6 × 6 cm piece of Reeve Angel SA-2 cation-exchange resin loaded paper. The paper is soaked in 20–50 ml of fresh undiluted urine and shaken for 20–30 min on a reciprocating shaker. The cation-exchange resin paper is then removed and transferred to a 4-oz. wide-mouthed screw-capped jar. The spent urine is saved for acid hydrolysis of benzodiazepines and morphine glucuronides. The ion-exchange paper is rinsed with 10 ml of water (the rinsings being discarded) and extracted at pH 10.1 using ammonium chloride–ammonium hydroxide buffer and chloroform–isopropanol (5:2) as described earlier[32–36]. The aqueous buffer phase is saved and used for the extraction of benzoylecgonine.

(b) Benzoylecogonine (major cocaine metabolite). The aqueous buffer phase (remaining after pipetting the lower organic phase) containing the ion-exchange paper is adjusted to pH 1–2 by the addition of concentrated hydrochloric acid and then

saturated with sodium hydrogen carbonate. Benzoylecgonine is extracted with chloroform–isopropanol–dichloroethane as described elsewhere[36].

(c) Acid hydrolysis of spent urine for benzodiazepines and morphine glucuronide. The spent urine saved above (Procedure II(a)) is transferred to a 4-oz. wide-mouthed screw-capped jar, 5 ml of concentrated hydrochloric acid are added and the contents swirled. The method as described under Procedure I is followed from "The jar is then placed in an oven maintained at 100° for 1 h".

Procedure III. Acid hydrolysis of spent urine using Jet Cloth inert fibrous matrix[30]

Alternatively, the spent urine saved above (Procedure II(a)) is transferred to a 4-oz. wide-mouthed screw-capped jar, a 12 × 13-in. piece of inert fibrous matrix* is added to the urine. The fibrous matrix is kept soaked for 20–30 min with intermittent shaking, the unabsorbed urine is discarded and the bottle is kept in an inverted position on a paper towel to drain off the excess urine from the fibrous matrix. A 30-ml volume of deionized water and 5 ml of concentrated hydrochloric acid are added to the bottle containing the inert fibrous matrix. The contents are mixed with a stirring rod and then the method as described under Procedure I is followed from "The jar is then placed in an oven maintained at 100° for 1 h".

Thin-layer chromatography (TLC)

Gelman pre-coated silica gel glass microfiber sheets (ITLC, Type SA) with a layer thickness of 250 μm were used throughout.

Developing solvents. A special solvent is used for benzoylecgonine and different solvent systems (C, D, E and F) are used for poly-drugs as reported earlier by Kaistha and co-workers[32–36]. For benzodiazepines, cyclohexane–acetone (90:10) is used as the solvent.

Detection reagents. For benzodiazepines the following reagents are used:

(1) sodium nitrite, 0.4% (w/v) solution in water; use a fresh solution;

(2) sulfuric acid, 5% (v/v) solution in water;

(3) N-1-naphthylethylenediamine dihydrochloride, 0.2% (w/v) in methanol; use a fresh solution.

For poly-drugs and benzoylecgonine, the detection reagents used are as described by Kaistha and co-workers[32–36].

Detection procedures

Procedure A, for benzodiazepine benzophenones. The residue** obtained as described under Procedure I or II(c) or III is dissolved in 30–50 μl of methanol, vortexed and spotted on a 20 × 20 cm Gelman pre-coated silica gel glass microfiber sheet (Gelman ITLC, Type SA). One or two capillaries of ACB and MACB standards, each having a concentration of 1 mg/ml in methanol, are spotted along with the urine

* A 24 × 26-in. piece of inert fibrous matrix without extraction column (Product No. 3020), obtainable from Manhattan Instruments, 1304 Olympic Boulevard, Santa Monica, Calif. 90404, U.S.A., is cut into two equal halves. Product No. 3020 is a disposable JET tube packed with an inert fibrous matrix of large surface area.

** This residue can also be used for the detection of morphine glucuronide. On hydrolysis morphine is liberated from its glucuronide and extracted at pH 10.1. A portion of this residue can be spotted separately and developed in solvent E or C[32–36] and morphine can be detected by spraying with iodoplatinate reagent.

specimens. The spots are air dried and the plate is then dried for 5 min in an oven maintained at 85–90° before developing it in 100 ml of benzodiazepine solvent. The plate is removed from the solvent after the latter has travelled a distance of about 14.5–15.0 cm (travelling time 40–45 min). The plate is then air dried for 10 min and examined visually for the presence of MACB as a light yellow or yellow spot. The yellow spots at the level of MACB and ACB standards are circled (the R_F values are about 0.73–0.79 for MACB and 0.43–0.47 for ACB). Detection reagents 1–3 (see below) are applied in succession to the whole plate.

(1) *Sodium nitrite.* The plate is sprayed with sodium nitrite solution, kept at room temperature for 5 min and then examined under longwave UV light. Bluish-purplish fluorescent spots observed at the level of MACB standard are circled (MACB reference standard may show an additional minor spot at $R_F = 0.37$).

(2) *Sulfuric acid.* On the application of this spray, the yellow spots seen visually prior to and after spraying with sodium nitrite disappear. The plate is examined under longwave UV light after 5 min and bluish-purplish fluorescent spots at the level of MACB and ACB standards are circled (ACB reference standard may show two additional minor spots at of $R_F = 0.22$ and 0.34).

(3) *N-1-Naphthylethylenediamine dihydrochloride.* This spray is applied heavily; ACB gives a purple spot immediately (additional minor spots observed under longwave UV light do not respond to this spray)*. MACB and its additional minor spot do not give any purple color immediately; concentrations higher than 5 μg may give a light purple coloration.

Procedure B, for poly-drugs. The residue obtained as described under Procedure II(a) is dissolved in 30–50 μl of methanol, vortexed and the entire extract is spotted on a 20 × 20 cm Gelman pre-coated silica gel glass microfiber sheet (ITLC, Type SA). Using a two-stage development solvent system, the entire array of drugs of abuse is detected, as reported earlier by Kaistha and co-workers[32–36].

Procedure C, for benzoylecgonine. The residue obtained as described under Procedure II(b) is dissolved in 30–50 μl of dichloroethane, vortexed and spotted on a 20 × 20 cm Gelman pre-coated silica gel glass microfiber sheet (ITLC, Type SA). The plate is heated in the oven maintained at 85–90° for 5 min and developed in 100 ml of benzoylecgonine solvent (chloroform–methanol–water–concentrated ammonia solution, 70:30:0.5:1). Benzoylecgonine is detected as described earlier by Kaistha and Tadrus[36].

RESULTS AND DISCUSSION

Figs. 1 and 2 depict the metabolism of chlordiazepoxide and diazepam. The proposed detection procedures involving acid hydrolysis of raw urine are based on the identification of 2-amino-5-chlorobenzophenone (ACB, IV) and 2-methylamino-5-chlorobenzophenone (MACB, IX). Acid hydrolysis of chlordiazepoxide and/or its metabolites and oxazepam and/or its glucuronide will generate ACB. Oxazepam glucuronide is a common metabolite in the biotransformation of oxazepam (Serax), chlordiazepoxide (Librium), diazepam (Valium) and chlorazepate (Tranxene). Although ACB is yellow, minute concentrations of it do not show as yellow spots but

* A yellow spot alone, seen visually at the level of MACB without the concurrent presence of the purple spot of ACB is not indicative of benzodiazepine usage.

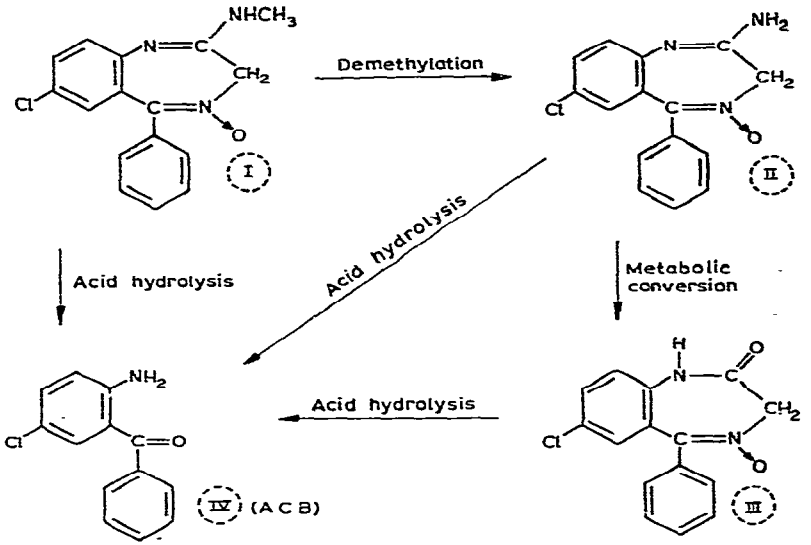

Fig. 1. Metabolism and chemical reactions of chlordiazepoxide.

can be readily detected by spraying with Bratton–Marshall reagent[16]. ACB gives purple spots of varying intensity after spraying with Bratton–Marshall reagent. Acid hydrolysis of 3-hydroxydiazepam and/or its glucuronide, the additional urinary metabolite of diazepam (Valium), will generate MACB. This benzophenone can be seen as a light yellow to yellow spot after TLC separation of the extracted residue. The concurrent presence of the upper MACB spot at an R_F value of approximately 0.73–0.79 and of the lower ACB spot at an R_F value of about 0.43–0.47 leads to the positive identification of the usage of diazepam (Valium). The presence of ACB alone indicates the usage of chlordazepoxide (Librium), oxazepam (Serax) and chlorazepate (Tranxene). Specificity of detection of ACB has been achieved by using a thin-layer developing solvent system (cyclohexane–acetone, 90:10) in which sulfonamides with primary aromatic amino groups remain at the origin, thus eliminating false positive results.

Several controlled studies validating the efficacy of the proposed detection procedures were conducted on 18–24-h pooled urine after oral ingestion of the drug. The dosages ingested were a 5-mg diazepam (Valium) tablet once a day and twice a day; and a 10-mg tablet once a day and thrice a day (each 10-mg tablet was ingested at 4-h intervals).

A 20–50-ml aliquot of pooled urine obtained from each of the above-controlled administrations was carried through all three proposed detection procedures. Procedures I (direct acid hydrolysis of urine) and II(c) (direct acid hydrolysis of spent urine) in all instances showed both MACB and ACB, while procedure III (acid hydrolysis of spent urine using inert fibrous matrix) showed ACB in all instances but MACB only after ingestion of a total of 30 mg of diazepam. Therefore, Procedures I and II(c) proved to be more reliable than Procedure III for the detection of diazepam (Valium). A controlled study on a 21-h pooled urine after the ingestion of a single dose of 25 mg of chlordiazepoxide was also conducted. A 20–50-ml aliquot of pooled urine was carried through each of the procedures, and ACB was detected in each instance.

A feasibility study of the use of cation-exchange resin loaded paper to test the

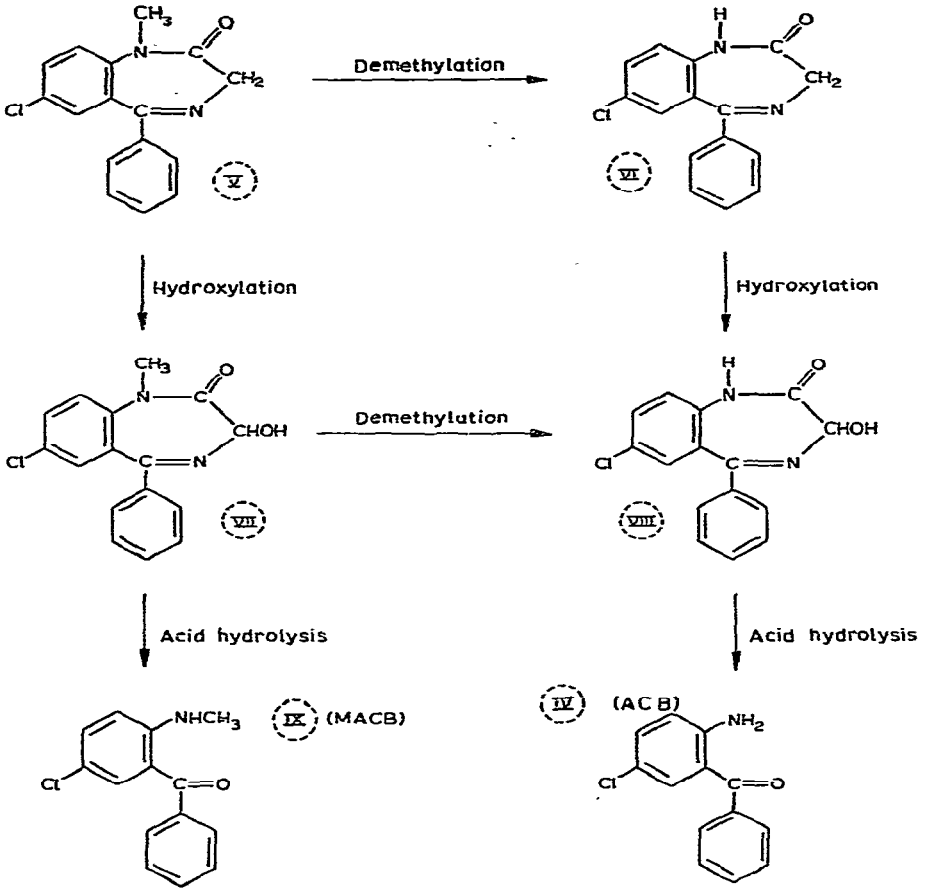

Fig. 2. Metabolism and chemical reactions of diazepam.

presence of benzodiazepine drugs was also conducted. A 40–50-ml aliquot of pooled urine obtained from each of the above controlled administrations was shaken for 30 min with a 6 × 6 cm piece of cation-exchange resin loaded paper. The paper was then removed and hydrolysed with concentrated hydrochloric acid as described under Procedure III using inert fibrous matrix. No MACB or ACB was detected, which proved the absence of free metabolites in amounts sufficient to generate measurable amounts of MACB and/or ACB. However, 20 out of 24 specimens of urines collected from 20 different subjects in a methadone maintenance program, when processed using acid hydrolysis of cation-exchange resin loaded paper as described above, showed the presence of MACB and/or ACB (more than half showed only ACB). Urine specimens collected from a subject who was not on a methadone maintenance program but has been using diazepam on a long-term basis showed the presence of both MACB and ACB using the ion-exchange paper technique. The authors are of the opinion that subjects on long-term usage of diazepam do excrete appreciable amounts of free metabolites capable of generating detectable amounts of ACB on acid hydrolysis of ion-exchange paper.

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REFERENCES

1 K. K. Kaistha, *J. Chromatogr.*, 141 (1977) 145.
2 *Comprehensive Product Information*, Professional Services Bulletin Nos. 0275B, 1275A and 0875A, Hoffmann-La Roche, Nutely, N.J., U.S.A.
3 B. A. Koechlin, M. A. Schwartz, G. Krol and W. Oberhansli, *J. Pharmacol. Exp. Ther.*, 138 (1965) 399.
4 M. A. Schwartz and E. Postma, *J. Pharm. Sci.*, 55 (1966) 1358.
5 M. A. Schwartz, E. Postma and Z. Gaut, *J. Pharm. Sci.*, 60 (1971) 1500.
6 M. A. Schwartz and E. Postma, *J. Pharm. Sci.*, 61 (1972) 123.
7 R. Dixon, M. A. Brooks, E. Postma, M. R. Hackman, S. Spector, J. D. Moore and M. A. Schwarzt, *Clin. Pharmacol. Ther.*, 20 (1976) 450.
8 J. A. F. de Silva, Hoffmann-La Roche, Nutley, N.J., personal communication; M. A. Brooks, L. d'Arconte, M. R. Hackman and J. A. F. de Silva, *Anal. Toxicol.*, (1977) in press.
9 H. B. Kimmel and S. S. Walkenstein, *J. Pharm. Sci.*, 54 (1967) 538.
10 B. A. Koechlin and L. D'Arconte, *Anal. Biochem.*, 5 (1963) 195.
11 N. Strojny, K. Bratin, M. A. Brooks and J. A. F. de Silva, *J. Chromatogr.*, 143 (1977) 363.
12 A. Zingales, *J. Chromatogr.*, 61 (1971) 237.
13 F. L. Vandemark and R. F. Adams, *Abstr. 28th Nat. Meet. Amer. Ass. Clin. Chem., Houston, Texas, Aug. 1–6, 1976*, p. 55.
14 D. J. Allison, B. G. Stephens and C. H. Hine, *Abstr. 29th Annu. Meet. Amer. Acad. Forensic Sci., San Diego, Calif., Feb. 1977*, p. 121.
15 L. O. Randall, *Dis. Nerv. Syst.*, 22 Suppl. 1 (1961) 7.
16 A. C. Bratton and E. K. Marshall, Jr., *J. Biol. Chem.*, 128 (1939) 537.
17 R. J. Kokoski, S. Hamner and M. Shiplet, *5th Nat. Conf. Methadone Treatment, Washington, D.C., March 17–19, 1973.*
18 S. A. Kaplan, M. L. Jack, K. Alexander and R. E. Weinfeld, *J. Pharm. Sci.*, 62 (1973) 1789.
19 D. J. Greenblatt and R. I. Shader, *Benzodiazepines in Clinical Practice*, Raven Press, New York, 1974, pp. 7 and 24.
20 J. A. F. de Silva and C. V. Puglisi, *Anal. Chem.*, 42 (1970) 1725.
21 W. Schallek, W. Schlosser and L. O. Randall, *Advan. Pharmacol. Chemother.*, 10 (1972) 119.
22 N. W. Blacow (Editor), *Martindale: The Extra Pharmacopoeia*, Pharmaceutical Press, London, 26th.ed., 1972, p. 1823.
23 M. A. Schwartz, B. A. Koechlin, E. Postma, S. Palmer and G. Krol, *J. Pharmacol. Exp. Ther.*, 149 (1965) 423.
24 J. A. F. de Silva, B. A. Koechlin and G. Bader, *J. Pharm. Sci.*, 55 (1966) 692.
25 J. A. F. de Silva, M. A. Schwartz, V. Stefanovic, J. Kaplan and L. D'Arconte, *Anal. Chem.*, 36 (1964) 2099.
26 J. M. Meola and H. R. Brown, *Abstr. 28th Nat. Meet. Amer. Ass. Clin. Chem., Houston, Texas, Aug. 1–6, 1976*, p. 55.
27 B. H. Haden, K. G. McNeil, N. A. Huber, W. A. Khan, P. Sing and R. S. Schneider, *Abstr. 28th Nat. Meet. Amer. Ass. Clin. Chem., Houston, Texas, August 1–6, 1976*, p. 57.
28 K. Grant, *Hosp. Pharm.*, 12 (1977) 352, and references cited therein.
29 *Physician's Desk Reference*, Medical Economics Co., Oradell, N. J., 31st ed., 1977, p. 548.
30 *Jet Tube Applications, Bulletin 3DS3020*, Manhattan Instruments, Santa Monica, Calif.
31 K. K. Kaistha and J. H. Jaffe, *J. Chromatogr.*, 60 (1971) 83.
32 K. K. Kaistha and J. H. Jaffe, *J. Pharm. Sci.*, 61 (1972) 679.
33 K. K. Kaistha, R. Tadrus and R. Janda, *National Drug Abuse Conference, Chicago, Ill., March 30th–31st, 1974.*
34 K. K. Kaistha, R. Tadrus and R. Janda, *J. Chromatogr.*, 107 (1975) 359.
35 K. K. Kaistha and R. Tadrus, *Clin. Chem.*, 22 (1976) 1936.
36 K. K. Kaistha and R. Tadrus, *J. Chromatogr.*, 135 (1977) 385.