complexed drug noticed a decrease in severity or no headache at all with the combination sample. This response can be explained from the recovery curve for the combination samples, since it was unlikely that development of tolerance to the stimulant occurred.

Table III shows the amount of amphetamine recovered in the urine over 48 hr and also the bioavailability of amphetamine from the complex and combination samples. When using the Student paired t test, no significant differences were seen in the bioavailability of amphetamine from all three formulations. However, excretion rates of amphetamine from the 1:20 complex were significantly different from the pure drug during the initial 4 hr.

In conclusion, the drug–excipient interaction between cationic drugs and montmorillonite clay can be employed successfully to prolong the action of such medicinals. The peak urinary excretion rate of amphetamine from the 1:20 drug–clay complex occurred at a much later time than the pure drug, indicating delayed absorption of amphetamine from the complex. In addition, the initial excretion rate of amphetamine from the complex was significantly lower than that of the pure drug. Finally, for all subjects, the bioavailability of the amphetamine from the 1:20 complex and the amphetamine–1:20 complex combination formulations over 48 hr was not significantly different from that of the pure drug.

REFERENCES

(1) D. C. Monkhouse and J. L. Lach, *Can. J. Pharm. Sci.*, **7**, 29(1972).

(2) J. W. McGinity and J. L. Lach, *J. Pharm. Sci*, **65**, 896(1976).

(3) K. Munzel, *Acta Pharmacol. Toxicol., Suppl. 3*, **29**, 81(1971).

(4) A. B. Varley, *J. Am. Med. Assoc.*, **206**, 1745(1968).

(5) M. J. Akers, J. L. Lach, and L. Fischer, *J. Pharm. Sci.*, **62**, 391(1973).

(6) J. G. Wagner, P. G. Welling, K. P. Lee, and J. E. Walker, *ibid.*, **60**, 666(1971).

(7) J. W. Bettis, J. L. Lach, and J. Hood, *Am. J. Hosp. Pharm.*, **30**, 240(1973).

(8) A. H. Beckett and M. Rowland, *J. Pharm. Pharmacol.*, **17**, 59(1965).

(9) A. H. Beckett, J. A. Salmon, and M. Mitchard, *ibid.*, **21**, 251(1969).

(10) G. A. Alles and B. B. Wisegarver, *Toxicol. Appl. Pharmacol.*, **3**, 678(1961).

(11) R. T. Williams, in "Fundamentals of Drug Metabolism and Drug Disposition," B. N. La Du, H. G. Mandel, and E. L. Way, Eds., Williams & Wilkins, Baltimore, Md., 1972, pp. 189–191.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 19, 1975, from the *College of Pharmacy, University of Iowa, Iowa City, IA 52240*

Accepted for publication March 8, 1976.

[x] To whom inquiries should be directed. Present address: College of Pharmacy, University of Texas at Austin, Austin, TX 78712

Mass Screening and Confirmation of Codeine and Morphine in Urine by Radioimmunoassay–GLC

NARESH C. JAIN *‡[x], THOMAS C. SNEATH ‡, WAI J. LEUNG ‡, and ROBERT D. BUDD ‡

Abstract □ A rapid, sensitive, and specific procedure is described for the mass screening and confirmation of codeine and morphine in urine specimens. The method is sensitive to 0.5-μg/ml levels of both opiates in free and/or conjugate forms. The raw urine is screened directly by radioimmunoassay, which is reactive to both free and glucuronide forms of codeine and morphine. Specimens that are screened positive are confirmed by GLC using a flame-ionization detector. The opiates are analyzed as their acetyl derivatives on two different columns, OV-25 and Poly-A 103. This multiple approach eliminates false positives caused by interfering substances or structurally similar compounds present in the urine.

Keyphrases □ Codeine—radioimmunoassay–GLC analysis, human urine □ Morphine—radioimmunoassay–GLC analysis, human urine □ Radioimmunoassay–GLC—analysis, codeine and morphine in human urine □ GLC—analyis, codeine and morphine in human urine □ Opiates—codeine and morphine, radioimmunoassay–GLC analysis in human urine □ Narcotics—codeine and morphine, radioimmunoassay–GLC analysis in human urine

The widespread use and abuse of codeine, morphine, and heroin, which is largely metabolized to morphine (1), necessitate the development of large-scale procedures for the determination of codeine and morphine in urine samples. A large urine drug testing laboratory must have a sensitive and specific method that can rapidly separate "true negative" from "presumptive positive" specimens. It is equally important to have a fundamentally different confirmatory method that can independently and accurately confirm the presence of an opiate and, at the same time, identify the individual drug. The method should also give quantitative results when needed. Since codeine and morphine are primarily excreted in the urine as conjugates in the form of glucuronides (1, 2), the methods of analysis must be able to detect them in both forms.

BACKGROUND

Most laboratories use TLC to screen urine specimens for various drugs including codeine and morphine (3, 4). The technique is relatively inexpensive but, on a large scale, has many disadvantages such as poor sensitivity, inconsistency in R_f values, variations with humidity, subjectivity in data interpretation, and the requirement of hydrolysis to detect the opiate glucuronides. Although the reagents and equipment for radioimmunoassay are expensive, the consistency of results, ability to detect glucuronide forms, increased sensitivity, greater accuracy, and semiautomation of the method make it well worth the cost involved (5–8).

Since free opiates have poor sensitivity by GLC, a number of GLC procedures have been published to separate and identify their derivatives (9–11). Most methods use only one column, which provides no assurance of avoiding false positives from interfering peaks with retention times similar to the opiate derivatives on that particular column.

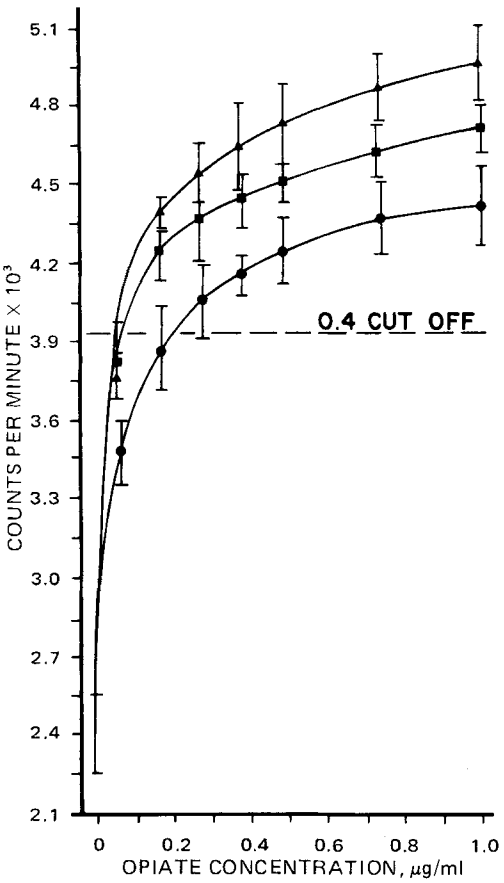

Figure 1—*Radioactive count curves for codeine (▲), morphine (●), and morphine glucuronide (■) with the morphine antibody. Standard deviations are included.*

This confirmation procedure is so designed that interfering substances are not reported as false positives. The opiates are analyzed as their acetyl derivatives on two quite different GLC columns: 3% OV-25 and 3% Poly-A 103. True positives must give the proper response on both columns.

Nalorphine is added to each urine specimen in the first step of extraction and is carried throughout the analytical process as an internal standard to ensure proper extraction and sample preparation. This procedure eliminates false negatives, allows accurate quantitation of positives, and assures correct results.

Several other opiates and drugs such as methadone, the primary metabolite of methadone (2-ethylidine-1,5-dimethyl-3,3-diphenylpyrrolidine), propoxyphene, norpropoxyphene, meperidine, apomorphine, dihydromorphine, hydrocodone, oxycodone, berberine (goldenseal), papaverine, and hydromorphine have been analyzed for possible interference with radioimmunoassay and/or GLC. With the procedure presented here, interference from any of these compounds is eliminated.

Table I—Relative Retention Times (RRT) of Acetylated Opiates and Other Drugs

Drug	Forms Acetyl Derivative[a]	RRT on 3% OV-25[b]	RRT on 3% Poly-A 103[c]
Codeine	Yes	0.50	0.46
Morphine	Yes	0.77	0.68
Nalorphine	Yes	1.00[d]	1.00[e]
Apomorphine	Yes	0.97	1.06
Dihydromorphine	Yes	0.82	0.91
Oxycodone	Yes	0.52	0.53
Hydromorphine	Yes	6.85	1.01[f]
Papaverine	No	1.86	2.35
Methadone	No	6.08	0.08
Methadone metabolite[g]	No	0.07	0.05
Propoxyphene	No	0.12	0.10
Norpropoxyphene	No	0.28	0.30[h]
Meperidine	No	0.03	0.03
Hydrocodone	Yes	0.49	0.60

[a] If "yes," relative retention times are for that derivative; if "no," relative retention times are for the parent drug. [b] Column temperature 250°; injector, 290°; detector, 300°; and carrier gas (nitrogen), 400 ml/min. [c] Column temperature, 230°; injector, 270°; detector, 280°; and carrier gas (nitrogen), 61 ml/min. [d] Retention time = 6.2 min. [e] Retention time = 7.3 min. [f] Second smaller peak with relative retention time of 0.70. [g] 2-Ethylidine-1,5-dimethyl-3,3-diphenylpyrrolidine. [h] Second smaller peak with retention time of 0.54.

EXPERIMENTAL

Materials—The following were obtained from commercial sources: morphine radioimmunoassay kit[1], ammonium sulfate[2], nalorphine hydrochloride[3], *tert*-butanol[2], chloroform[2], spectrograde chloroform[2], butyl acetate[2], dibasic potassium phosphate[2], potassium hydroxide[2], potassium carbonate[2], pyridine[2], anhydrous acetic anhydride[2], sodium hydroxide[2], hydrochloric acid[2], 3% Poly-A 103 on Gas Chrom Q[4] (100–120 mesh), and 3% OV-25 on HP Chrom W[5] (AW + DMCS) (80–100 mesh). All chemicals were reagent grade except as indicated.

Equipment—The following equipment was used: an automatic pipetting station[6], a centrifuge[7], a γ-scintillation counter with printer[8], a high-speed automatic pipet[9], 50-ml round-bottom centrifuge tubes with glass stoppers[10], 40-ml centrifuge tubes with tapered end[10], a dual-pen recorder[11], a gas chromatograph[12] equipped with dual flame-ionization detectors, and dual differential electrometers.

Radioimmunoassay Screening Procedure—The urine samples are screened for opiates using the Roche radioimmunoassay procedure (6) with slight modifications. Automatic pipetting stations are used for all dilutions; 0.40 ml of working reagent (morphine antigen–antibody 1:1) is added rather than 0.20 ml of each component separately.

A specimen is recorded as presumptive positive for opiates if its radioactive count is higher than the established cutoff value. The cutoff value is determined by checking all opiate controls run that day and selecting a level so that all 0.4-μg/ml controls of both opiates are detected. All specimens below the cutoff value are considered "negative." Specimens that give a radioactive count corresponding to 0.4 μg/ml or more of opiate are analyzed by GLC to confirm which particular drug is present.

GLC Confirmation Procedure—The urine samples screened positive by radioimmunoassay are confirmed as their acetylated derivatives by GLC using the procedure of Jain *et al.* (12).

Proper Sample Preparation—A peak for the internal standard (acetylated nalorphine) must appear on both GLC columns even when the specimen is negative for codeine and/or morphine. If the internal standard does not appear on a GLC column, the specimen has not been prepared properly and the procedure must be repeated.

Positive Specimen—A positive specimen for codeine and/or morphine must give the proper internal standard response and must produce acetylated codeine and/or morphine peaks of equal concentrations on both columns.

Negative Specimen—A negative specimen for codeine and/or morphine must give the proper internal standard response and must fail to give the proper acetylated codeine and/or morphine response on one or both GLC columns.

RESULTS AND DISCUSSION

The radioimmunoassay–GLC system is suitable for screening and confirming opiates in urine specimens on a large scale. The procedure can consistently detect 0.5-μg/ml levels of both free and conjugated forms of codeine and morphine.

The radioimmunoassay screening method is considered superior to other screening techniques, including TLC and the enzyme-multiplied

[1] Roche Diagnostics, Division of Hoffmann–La Roche, Nutley, N.J.
[2] Matheson, Coleman and Bell, Los Angeles, Calif.
[3] Merck Sharp and Dohme, West Point, Pa.
[4] Applied Science, State College, Pa.
[5] Varian Associates, Palo Alto, Calif.
[6] Model 24004, Micromedic Systems, Philadelphia, Pa.
[7] Model K with 418 head, International Equipment Co., Needham Heights, Mass.
[8] Model 5160, Packard Instrument Co., Los Angeles, Calif.
[9] Model 25004F, Micromedic Systems, Philadelphia, Pa.
[10] Corning Glass Works, Corning, N.Y.
[11] Model B2616 E, Soltec Corp., Encino, Calif.
[12] Model 2700, Varian Associates, Palo Alto, Calif.

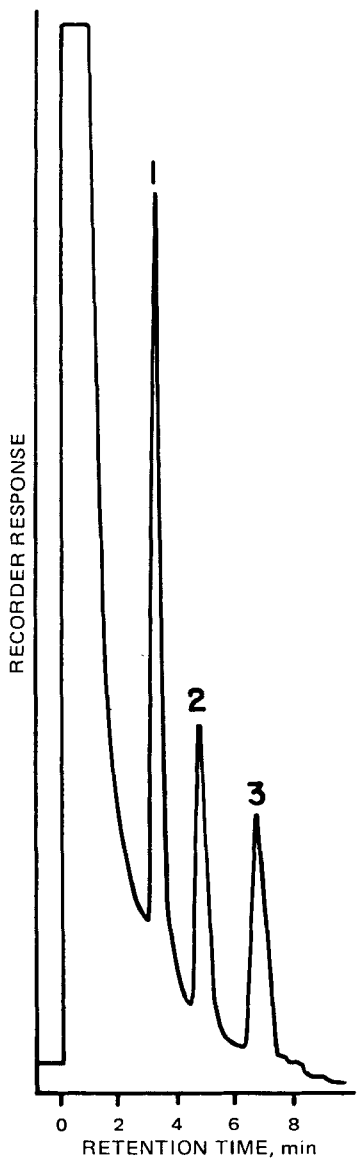

Figure 2—*Gas chromatogram of acetylated opiates on a 3% Poly-A 103 column (glass, 0.6 m × 2 mm). Operating conditions were: column, 230°; injector, 260°; and detector, 265°. Key: 1, codeine; 2, morphine; and 3, nalorphine.*

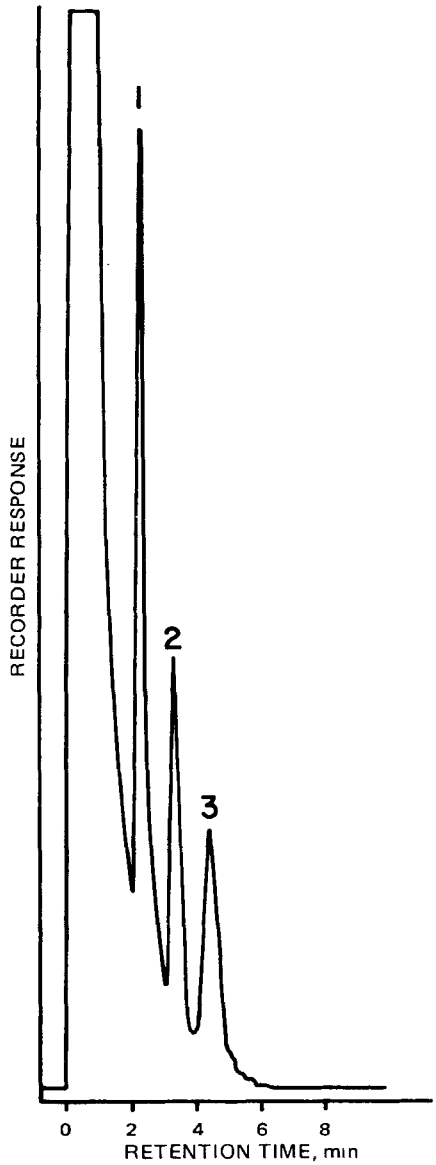

Figure 3—*Gas chromatogram of acetylated opiates on a 3% OV-25 column (glass, 0.9 m × 2 mm). Operating conditions were: column, 240°; injector, 280°; and detector, 285°. Key: 1, codeine; 2, morphine; and 3, nalorphine.*

immunoassay technique (13, 14). Both radioimmunoassay and the enzyme-multiplied immunoassay technique have much greater sensitivity, consistency, and accuracy of results than does TLC. They detect both free and glucuronide forms directly from raw urine and can be semiautomated so that one technician can screen many samples, depending upon the availability and type of equipment used. The radioimmunoassay antibody for opiates, however, is more sensitive than the enzyme-multiplied immunoassay technique reagents. For example, radioimmunoassay can detect morphine at a concentration of 0.1 μg/ml with greater than 99% accuracy (Fig. 1), whereas the enzyme-multiplied immunoassay technique requires a morphine concentration of 0.7 μg/ml to detect it 95% of the time (13, 14). The advantages of radioimmunoassay over other screening procedures make it preferable in spite of its increased cost.

As shown in Fig. 1, all of the opiates are not equally reactive to the radioimmunoassay antibody. As a result, the radioactive count cutoff value in radioimmunoassay must be based on the least reactive opiate to ensure that 0.5-μg/ml concentrations of all opiates of interest are detected. Contrary to earlier reports (6), morphine glucuronide is more reactive than free morphine to the opiate antibody.

Each specimen found positive by radioimmunoassay is confirmed by GLC. Since free opiates do not chromatograph well, codeine and morphine are confirmed by analyzing them as acetyl derivatives on two very different columns: OV-25 and Poly-A 103. The relative retention times of the opiates are presented in Table I. Figures 2 and 3 illustrate the clear separation obtained. The GLC confirmation method is designed so that, although the specimen is extracted only once, it is subjected to two parameters of analysis to obtain the most accurate and reliable results. This dual-column approach by GLC virtually eliminates false positive results. If GLC results for a sample require additional confirmation, the residue is spotted on a TLC plate[13], which is developed in ethyl acetate–acetone–concentrated ammonia (100:10:4). The R_f values for acetylated morphine, acetylated codeine, and acetylated nalorphine are 0.50, 0.65, and 0.83, respectively (15).

The use of nalorphine as an internal standard added at the beginning of the analysis not only allows the accurate quantitation of the opiates but also assures that samples are extracted properly. This procedure eliminates the possibility of reporting false negative results.

By using radioimmunoassay and both GLC columns, a series of spiked urine specimens containing varying concentrations of codeine, morphine, and morphine glucuronide, from 0.1 to 10 μg/ml of each, was analyzed. The data obtained showed above 99% accuracy at the 0.5-μg/ml level for each opiate. This error of less than 1% has always been due to borderline cases where a final result has been reported negative when, in fact, the sample

[13] Merck silica gel G, VWR Scientific, Los Angeles, Calif.

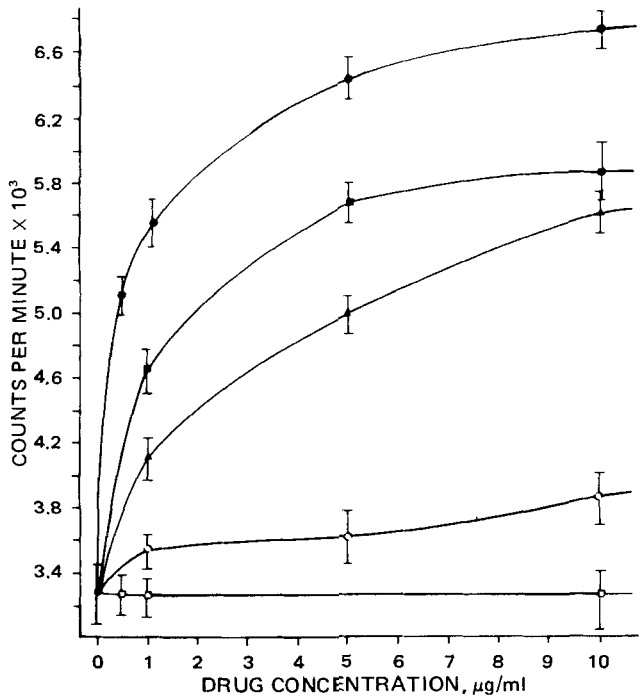

Figure 4—*Radioactive count curves for other drugs with the morphine antibody. Standard deviations are included. Key: ●, morphine; ■, dihydromorphine; ▲, heroin; ○, meperidine; and □, methadone, methadone metabolite, propoxyphene, norpropoxyphene, apomorphine, and papaverine.*

contained a very small amount of an opiate. These cases were detected by radioimmunoassay but, due to occasional variations in the extraction technique and flame-ionization detector response, the opiate gave a GLC response too small to be considered positive.

Urine samples spiked with various concentrations of methadone, methadone primary metabolite, propoxyphene, norpropoxyphene, apomorphine, dihydromorphine, hydrocodone, hydromorphine, heroin, papaverine, oxycodone, and berberine (goldenseal) also were tested. Heroin, dihydromorphine, hydromorphine, hydrocodone, oxycodone, and high concentrations of meperidine reacted with the radioimmunoassay opiate antibody (Fig. 4). All of these drugs could be differentiated from morphine, codeine, or nalorphine by the two GLC columns (Table I).

This radioimmunoassay–GLC method offers a sensitive and reliable confirmation procedure for ascertaining the presence or absence of codeine and morphine in urine on a large scale.

REFERENCES

(1) E. G. C. Clarke, "Isolation and Identification of Drugs," Pharmaceutical Press, London, England, 1969, pp. 164, 165, 269, 431, 432.

(2) J. T. Payte, J. E. Wallace, and K. Blum, *Curr. Ther. Res.*, **13**, 412 (1971).

(3) J. E. Wallace, J. D. Biggs, J. H. Meritt, H. E. Hamilton, and K. Blum, *J. Chromatogr.*, **71**, 135 (1972).

(4) J. Cochin and J. W. Daly, *Experientia*, **18**, 294 (1962).

(5) S. Spector and C. W. Parker, *Science*, **168**, 1347 (1970).

(6) "Abuscreen," Roche Diagnostics, Division of Hoffmann-La Roche, Nutley, N.J.

(7) S. J. Mulé, M. L. Bastos, and D. Jukofsky, *Clin. Chem.*, **20**, 243 (1974).

(8) S. J. Mulé, E. Whitlock, and D. Jukofsky, *ibid.*, **21**, 81 (1975).

(9) E. Schmezler, W. Yu, M. S. Hewitt, and I. J. Greenblatt, *J. Pharm. Sci.*, **55**, 155 (1966).

(10) J. E. Wallace, J. D. Briggs, and K. Blum, *Clin. Chem. Acta*, **36**, 85 (1972).

(11) F. Fish and W. D. Wilson, *J. Chromatogr.*, **40**, 164 (1969).

(12) N. C. Jain, R. D. Budd, and T. C. Sneath, in "CRC Methodology for Analytical Toxicology," I. Sunshine, Ed., CRC Press, Cleveland, Ohio, 1975, pp. 271–274.

(13) K. E. Rubenstein, R. S. Schneider, and E. F. Ullman, *Biochem. Biophys. Res. Commun.*, **47**, 846 (1972).

(14) "EMIT Opiate Assay," Syva Corp., Palo Alto, Calif., 1973.

(15) N. C. Jain, T. C. Sneath, R. D. Budd, and W. J. Leung, *Clin. Chem.*, **21**, 1486 (1975).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 22, 1975, from the *Departments of Pharmacology and of Community Medicine and Public Health, University of Southern California Medical School, Los Angeles, CA 90033, and the ‡Rancho Los Amigos Hospital, Downey, CA 90242.

Accepted for publication March 10, 1976.

ˣ To whom inquiries should be directed (at Rancho Los Amigos Hospital).

Use of Rabbits for GI Drug Absorption Studies

TADAO MAEDA ˣ, HIROSHI TAKENAKA, YOSHIYA YAMAHIRA, and TAKESHI NOGUCHI

Abstract □ A novel procedure to control the stomach emptying rate in rabbits is presented. Rabbits were given a special solid diet for 1 week, and then the gastric contents were washed out with saline. Then the rabbits were muzzled to prevent coprophagy during the night. Fifty grams of special soft diet given to the "stomach-emptying-controlled" rabbit transferred exponentially from the stomach into the small intestine and almost disappeared from the stomach within 5 hr. Griseofulvin, indomethacin, or nalidixic acid was administered in a hard gelatin capsule or tablet, with subsequent feeding of a special soft diet. Good correlations were observed between the plasma level–time curves of these drugs in the stomach-emptying-controlled rabbits and in human subjects.

Keyphrases □ Absorption, GI—griseofulvin, indomethacin, and nalidixic acid, effect of stomach emptying rate, rabbits □ GI absorption—griseofulvin, indomethacin, and nalidixic acid, effect of stomach emptying rate, rabbits □ Stomach emptying rate—effect on GI absorption of griseofulvin, indomethacin, and nalidixic acid, rabbits □ Griseofulvin—GI absorption, effect of stomach emptying rate, rabbits □ Indomethacin—GI absorption, effect of stomach emptying rate, rabbits □ Nalidixic acid—GI absorption, effect of stomach emptying rate, rabbits

In the study of GI absorption of drugs, various *in vitro* or *in situ* methods have been widely used (1–4). From the physiological point of view, the experimental animal data obtained by these destructive or operative techniques have only limited value in predicting drug absorption characteristics in humans, although such data provide funda-