CHROM. 6590

A NEW FLUORESCENCE ASSAY OF TRIMETHOPRIM AND METABOLITES USING QUANTITATIVE THIN-LAYER CHROMATOGRAPHY

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(Received December 12th, 1972)

SUMMARY

A new assay procedure using quantitative thin-layer chromatography has been developed for trimethoprim and its four major free and conjugated metabolites. The method is based upon the measurement of the fluorescence which develops for these compounds on non-fluorescing silica gel plates. Quantities as low as 0.03 μg can be determined. Previously reported assay procedures for trimethoprim have not been generally applicable for the assay of the metabolites. Accuracy and reproducibility data are reported for the determination of trimethoprim in urine, plasma and various tissues, and for the metabolites from urine.

INTRODUCTION

The combination* of trimethoprim (TMP)[1] with various sulfonamides, which are mutual potentiators[2], has provided a broad-spectrum antibacterial agent. A number of analytical methods for the assay of TMP in biological fluids have been reported. These include microbiological[3,4], spectrophotometric[3] and spectrofluorometric methods[5], and also scintillation spectrometry of ^{14}C-labeled TMP[6]. Only the latter method has been applied to the assay of both TMP and four metabolites, M1, M2, M3 and M4 (Fig. 1). Briefly, the ^{14}C-labeled metabolites were separated on silica gel plates, located either by fluorescence quench spectrofluorometry or by autoradiography, scraped from the plate, eluted, and radioactivity measured[6]. Although appropriate for initial studies, this method was not considered useful for monitoring blood and urine levels of drug and metabolites during clinical studies. Consequently, a non-radioactive and quantitative assay involving thin-layer chromatography (TLC) was investigated and developed for the assay of TMP and metabolites in biological fluids and tissues. The method is based upon the observation that after development on silica gel plates and subsequent exposure to light, this class of compounds develops a fluorescence which is proportional to concentration. The details of that procedure and its application to the assay of biological samples is the subject of this paper.

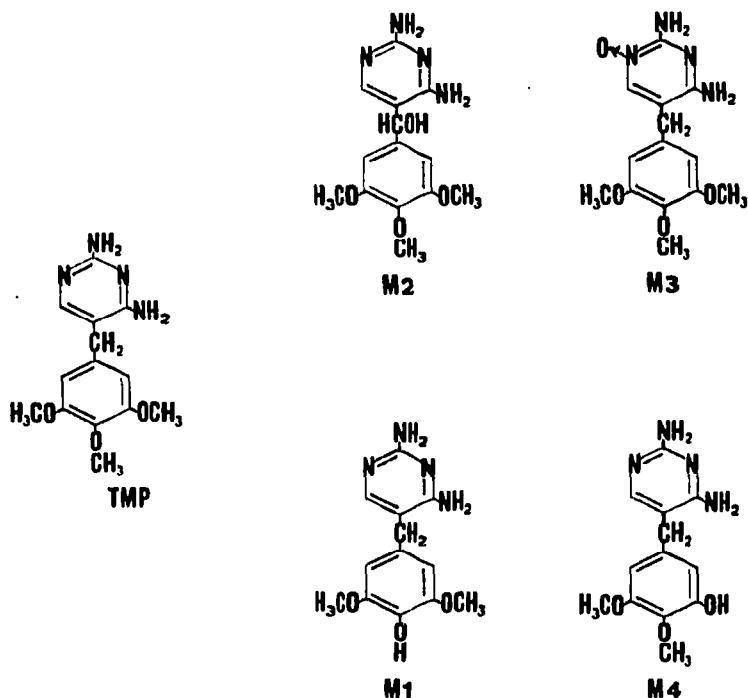* Trimethoprim–sulfamethoxazole (1:5 combination) called Septra®, Burroughs Wellcome.

Fig. 1. Trimethoprim and four metabolites which are numbered according to Schwartz et al.[6].

MATERIALS AND METHODS

Solvents and chemicals

The following solvent systems were used for TLC: (A) chloroform–n-propanol–28 % aq. NH$_4$OH (80:20:1)[6]; (B) chloroform–n-propanol–28 % aq. NH$_4$OH (100:10:1); (C) chloroform–n-propanol–28 % aq. NH$_4$OH (25:20:1); (D) benzene–abs. ethanol (7:3).

All solvents were supplied by Mallinckrodt (reagent grade) and all, except ammonia, were distilled before use. Glusulase (181,448 units glucuronidase, 84,178 units sulfatase/ml) was a product of Endo Laboratories, Inc.

Thin-layer plates

Thin-layer plates were Analtech Silica Gel G/MH (0.25 mm, 20 × 20 cm) plates, which were scored prior to sample application. Other silica gel plates can be used, but they must not contain fluorescent indicators.

Extraction of TMP from urine and plasma

An aliquot of urine (5 ml) was pipetted into a centrifuge tube and 5 ml water added. The solution was adjusted to near pH 10 with 1 N NaOH and extracted with three 10-ml portions of chloroform. The tube was rotated gently by hand twenty times. Occasional emulsions were broken by centrifugation. Each chloroform extract was pipetted as completely as possible. The combined extracts were evaporated with a stream of dry nitrogen at 35°. For extraction of TMP from plasma, the same procedure

was followed, except that isopropanol–dichloromethane (1:4) was used. The latter system seldom formed emulsions.

Extraction of free metabolites M2 and M3 from urine

Urine (5 ml) was adjusted to pH 2 with 1 N H_2SO_4, and extracted with two 10-ml portions of chloroform, which were discarded. The aqueous phase was adjusted to pH 13 with 0.4 N NaOH (10 ml) and the procedure for the extraction of TMP followed.

Extraction of conjugated metabolites M1 and M4 from urine

A urine sample (5 ml) was adjusted to near pH 6 with acetic acid and glusulase added (0.3 ml/5 ml urine). The solution was incubated at 37° for 18 h. The incubation solution was adjusted to pH 2 with 1 N H_2SO_4, diluted to 10 ml with water, and washed with two 10-ml portions of chloroform (as above). The aqueous phase was adjusted to near pH 7.8 with 1 N NaOH and was then extracted using the TMP procedure.

Extraction of TMP from tissues

The tissue (about 1 g) was minced thoroughly with scissors and homogenized in 5 ml 0.2 N H_2SO_4 using a Duall tissue grinder. The grinder was washed with 5 ml 0.2 N H_2SO_4, which was combined with the original homogenate. This suspension was centrifuged (15,000 r.p.m., 20 min), an aliquot (9 ml) removed and the pellet resuspended in 8 ml 0.2 N H_2SO_4. After centrifugation, another aliquot (8 ml) was removed and combined with the first. The combined aqueous extracts were adjusted to pH 10 with 5 N NaOH and were then extracted. The extract was evaporated as for TMP.

Extraction of reference compounds for use as standards

For each series of unknown samples, aliquots of control urine were spiked with an appropriate range of concentrations of synthetic reference compounds. One series of standards was prepared for TMP and the free metabolites. A separate series using control urine previously treated with glusulase was prepared for the phenolic metabolites. These standards were extracted in the same manner as the unknowns. The chloroform extracts were evaporated with a stream of dry nitrogen, and the residue applied to TLC plates as outlined below.

TLC procedure

Residues from the chloroform extraction, unknowns and reference samples, were dissolved in methanol–chloroform (1:9), and an aliquot (2–40 μl) applied to a prescored silica gel plate. Solvent systems used for each determination are listed in Table I. After development to 15 cm, the plate was removed from the solvent tank and allowed to air dry with exposure to room light. For each compound a light-blue fluorescence, visible under long wavelength (360 nm) ultraviolet (UV) light, developed. Plate concentrations of TMP and metabolites above 0.2 μg could be scanned in about 24 h. For lower concentrations, two to five days were required for the intensity of the fluorescence to reach a sufficient level for quantitation. The plates were scanned with a Schoeffel Model SD 3000 spectrodensitometer using a reflectance mode with irradiation of the plate at 355 nm. Optical density was recorded on a Honeywell Electronik 194 recorder. All peaks were symmetrical and well resolved. Peak areas were integrated

TABLE I

TLC SEPARATION DATA FOR TMP AND METABOLITES

Compound	Solvent system*	Approximate R_F values
TMP	A (1)	0.56
M1	B (3)	0.55
M2	B (2) A (1)	0.58
M3	C (1)	0.28
M4	B (3)	0.32

* Parentheses indicate the number of times the plate was developed to 15 cm in each solvent system.

with a Hewlett-Packard Calculator, Model 9100 B. Standard curves were calculated using the method of least squares.

RESULTS AND DISCUSSION

The present method provides a procedure for measuring independently the concentrations of TMP and metabolites in urine. Generally two aliquots of urine are used. One sample is extracted directly for unchanged drug and free metabolites, and the other treated with glusulase. The residue from the first extract is redissolved in a known volume and three aliquots removed. Two are spotted on separate TLC plates for the assay of M2 and M3, respectively, and the third is diluted and used for the TMP assay. The TMP aliquot must be diluted since it is present in much higher concentrations than the metabolites. The phenolic metabolites, M1 and M4, are extracted from the hydrolyzed urine sample, and assayed on one TLC plate. Plates are developed using the solvent systems listed in Table I.

All extractions from urine are done with chloroform. Since that solvent does not extract the metabolites quantitatively, a correction for the partition is necessary. For each series of samples, control urines are spiked with an appropriate range of metabolite concentrations, and these standards are extracted and spotted on the plates in the same way as the unknowns. Each series of unknowns is calculated on the basis of a least squares line based upon from five to seven points corrected for the extraction procedure. Solvents more polar than chloroform were tried for the extraction, but these removed too many endogenous fluorescent compounds. Trimethoprim is extracted quantitatively and a correction is not necessary.

Typical standard curves for TMP and metabolite M3 are shown in Figs. 2 and 3, respectively. The practical lower limit for quantitation was found to be about 0.03 μg for each of the compounds, but amounts as low as 0.01 μg could be detected. A linear relationship between integrated peak area and concentration was observed between the lower limit of sensitivity and about 0.8 μg for each compound. Reproducibility and recovery data are shown in Table II.

The TLC method has been applied to the assay of TMP in plasma and various tissues and presumably could be extended to the assay of metabolites in these samples also. The only significant difference between the procedure for extraction of TMP from urine and plasma was that isopropanol–dichloromethane (1 :4) was used as the
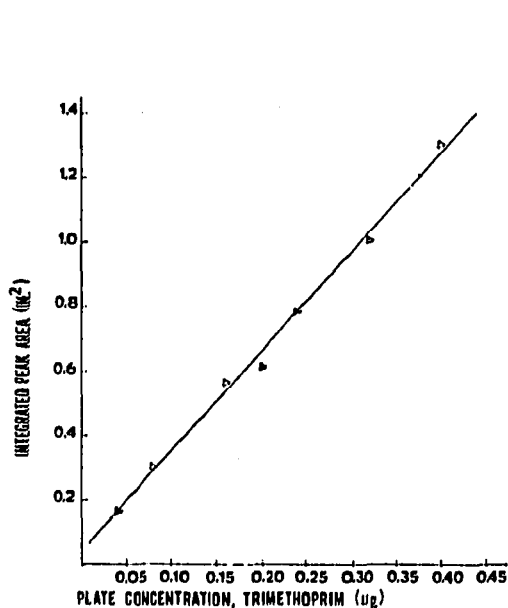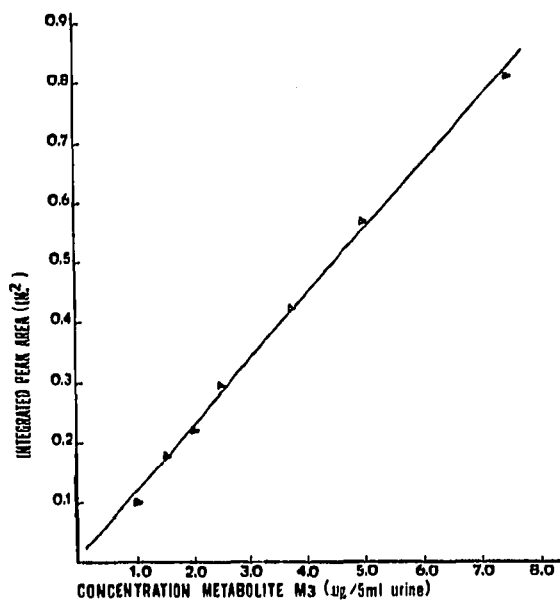
Fig. 2. Standard curve for TMP.

Fig. 3. Standard curve for extraction of M3 from urine.

solvent in the latter case. It afforded a higher recovery (greater than 95%) and seldom formed an emulsion.

The extraction prodecure of TMP from tissues outlined in the experimental section afforded high recoveries from several spiked tissues (see Table II).

The method outlined above has several advantages over previously reported methods. Unchanged drug is determined directly as a peak well separated from the metabolites. The microbiological assay methods are based upon total activity and some of the metabolites contribute to the activity.

The spectrophotometric methods include measurement of metabolites to some

TABLE II

REPRODUCIBILITY OF RECOVERY OF TMP AND METABOLITES FROM SPIKED PLASMA, URINE, AND VARIOUS TISSUES

Compound	Biological sample	Number of samples	Quantity added (μg)	Mean recovery	Percent recovery	Standard deviation
TMP	Urine (5 ml)	5	150	149	99	3.5
TMP	Plasma (1 ml)	6	1.2	1.18	98	0.04
TMP	Liver (1 g)	4	2.0	1.87	93	0.14
TMP	Prostate (1 g)	5	2.0	1.93	96	0.21
TMP	Lung (1 g)	4	2.0	1.62	81	0.06
M1	Urine (5 ml)	5	3.5	3.47	99	0.20
M2	Urine (5 ml)	5	7.5	7.25	96	0.52
M3	Urine (5 ml)	5	8.0	8.17	104	0.46
M4	Urine (5 ml)	5	4.5	4.43	98	0.20

extent also. With the TLC procedure, TMP and the metabolites are determined by specific and sensitive assays, and the complications introduced by radioactivity are avoided.

The techniques involved can easily be learned and the time required to prepare samples is not great. For very low concentrations, however, time is required for development of the fluorescence. TMP concentrations for fifteen to twenty samples can be determined in about twelve hours' technician time.

The method does not appear to be applicable to other diaminopyrimidines; however, Simmons and DeAngelis in these laboratories have found a quantitative TLC method which is applicable to a large number of diaminopyrimidines with the exception of the TMP type[7].

The source of the fluorescence which develops for each of these compounds has been investigated, but the identities of the fluorescent species have not been determined. The fluorescence developed on all commercial silica gel plates tried, with or without binders or fluorescent indicators. The rate of development, however, varied significantly for different plates. Analtech G/MH afforded measurable fluorescence the fastest. Light was necessary, but an optimum wavelength was not found. Laboratory light was supplied by diffuse fluorescent fixtures. Irradiation in the UV range (254–360 nm) did not increase the rate of development. Attempts to isolate fluorescent derivatives were unsuccessful. These studies did reveal that the fluorescence which develops for TMP is due to more than one compound. These materials account for only a small percentage of the TMP on the plate and their identification is a subject for future study.

Finally, as drug interactions and factors affecting metabolism in man become increasingly more important areas of research in the course of evaluating new and effective chemotherapeutic agents, new direct sensitive nonradioactive assay techniques are becoming more essential. Quantitative fluorescence TLC has provided a convenient tool of adequate sensitivity for TMP for continued studies in these areas.

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