Table I—Comparison of the *In Vitro* Relative Flux of Naproxen Using Excised Rat Skin and the Normalized Relative Rates of Percutaneous Absorption in the *In Vivo* Rat

Formulation	*In Vitro* Relative Flux[a]	*In Vivo* Normalized Relative Rates[b]
No added surfactant (control)	1	1
Sodium lauryl sulfate (2%)	1.3	1.5
Sodium laurate (2%)	1.7	1.8
Methyldecyl sulfoxide (1%)	1.5	2.8

[a] Data from Table II, Ref. 6. [b] Slopes of the linear regression lines in Fig. 2 were normalized.

It is also apparent that reliance on the rat using either *in vitro* or *in vivo* models may lead to erroneous conclusions regarding the effect of surfactants on human percutaneous absorption of drugs similar to naproxen. A great degree of caution should be exercised in using animal bioassay models in evaluating the effect of formulation changes on topical absorption.

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Rapid GLC Determination of Chlordiazepoxide and Metabolite in Serum Using On-Column Methylation

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Abstract □ A rapid GLC method was developed for the assay of chlordiazepoxide in serum. After chlordiazepoxide was extracted with ether, it was methylated with trimethylanilinium hydroxide in the injection port and detected by electron capture. The assay is simple and sensitive and can be automated for large-scale clinical analysis.

Keyphrases □ Chlordiazepoxide—electron-capture GLC analysis in serum □ GLC, electron capture—analysis, chlordiazepoxide in serum □ Tranquilizers—chlordiazepoxide, electron-capture GLC analysis in serum

Electron-capture GLC determination of chlordiazepoxide in biological fluids was accomplished by assaying for either its hydrolysis product (1, 2) or the intact moiety (3, 4). These procedures were somewhat tedious and required large amounts of sample. The following method is rapid and has a sensitivity of about 45 ng/ml using 0.4 ml of serum.

EXPERIMENTAL

Reagents—Chlordiazepoxide hydrochloride[1] (I), trimethylanilinium hydroxide[2] in methanol (0.1 *M*), and 1,3-dimethyl-6-methoxy-4-(*p*-chlorophenyl)-1*H*-pyrazolo[3,4-*b*]pyridine[3] (II) as the internal standard were used as supplied. Ether[4] was anesthetic grade, and other chemicals were analytical reagent grade.

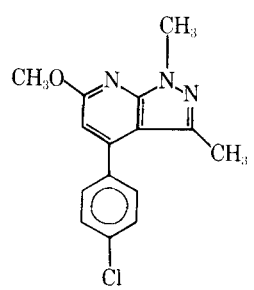
Instrumentation—A gas chromatograph[5], equipped with a ^{63}Ni-electron-capture detector containing a 2-mCu ^{63}Ni-β-ionization source, and an electronic integrator were used. The glass column was 1.21 m (4 ft) × 4 mm (i.d.), packed with 5% OV-225[6] on 80–100-mesh Gas Chrom Q[6]. The column was conditioned at 265° for at least 18 hr with a carrier gas [argon–methane[7] (95:5)] flow rate of 50 ml/min. The cylinder of the carrier gas was fitted with an oxygen trap filter[8].

The operating conditions were: column oven temperature, 265°; electron-capture detector temperature, 300°; injection port temperature, 270°; detector pulse interval, 150 μsec; electrometer range, 10^3; recorder presentation, 2 mv; and slope sensitivity, 0.3 mv/min. Under these conditions, the internal standard and chlordiazepoxide had retention times of 1.8 and 3.0 min, respectively (Fig. 1).

Table I—Assay Reproducibility

Serum Chlordiazepoxide Concentration, μg/ml	Calculated Serum Concentration[a], μg/ml	*RSD*, %
0	0	—
0.10	0.099 ± 0.0098	9.9
0.25	0.229 ± 0.062	27.2
0.75	0.758 ± 0.013	1.7
1.00	1.04 ± 0.14	13.7
1.50	1.48 ± 0.068	4.6

[a] Mean ± *SD* of triplicate determinations at each concentration.



II

[1] Lot 351074, Hoffmann–La Roche, Nutley, N.J.
[2] Eastman Kodak Chemicals, Rochester, N.Y.
[3] Lot 3827-LRS-25, synthesized from methylation of Compound 22 in *J. Heterocycl. Chem.*, **12**, 1137 (1975).
[4] Mallinckrodt Chemicals, St. Louis, Mo.
[5] Hewlett-Packard model 7620A.
[6] Applied Science Laboratories, State College, Pa.
[7] Matheson Gas Products, Elk Grove Village, Ill.
[8] Altech Associates, Arlington Heights, Ill.

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Table II—Assay Sensitivity

Serum Chlordiazepoxide Concentration, μg/ml	Peak Area Ratio, Chlordiazepoxide to Internal Standard
0	0
0	0
0	0
0.10	0.09
0.10	0.11
0.10	0.11
0.25	0.23
0.25	0.34
0.25	0.20
0.50	0.55
0.50	0.56
0.75	0.88
0.75	0.87
0.75	0.90
1.00	1.29
1.00	1.35
1.00	1.03
1.50	1.72
1.50	1.82
1.50	1.66
Least-Squares Linear Regression Calculation (7)	
Slope	1.1841
y-Intercept ± 95% confidence limit	−0.0141 ± 0.0533
Correlation coefficient	0.993
Calculated sensitivity, μg/ml[a]	0.0450

[a] The calculated sensitivity was the x value (in concentration) corresponding to the upper part of the 95% confidence interval of the y-intercept (in peak area ratio).

Preparation of Standards—A serum standard at 1.5 μg/ml for chlordiazepoxide was prepared from a freshly prepared primary standard solution at 100 μg/ml in water. Other serum standards were prepared from serial dilutions (with serum) of the 1.5-μg/ml serum standard.

An internal standard solution at 3 μg/ml in 0.1 M K_2HPO_4 was prepared from an initial standard at 100 μg/ml in methanol.

Extraction Procedure—To a 15-ml conical glass centrifuge tube were added 1.0 ml of the internal standard solution, 0.4 ml of serum, and 3 ml of ether. After mixing[9] for 15 sec and centrifuging at 2500 rpm for 5 min at 10°, 2 ml of the organic layer (top) was transferred into a conical tube and evaporated to dryness at 40° with filtered air. The residue was reconstituted with 1 ml of 0.01 M trimethylanilinium hydroxide in methanol; after mixing, the solution was transferred into disposable GLC vials[10]. After the vial was capped, a 2-μl aliquot was injected into the gas chromatograph using an automatic sampler[11].

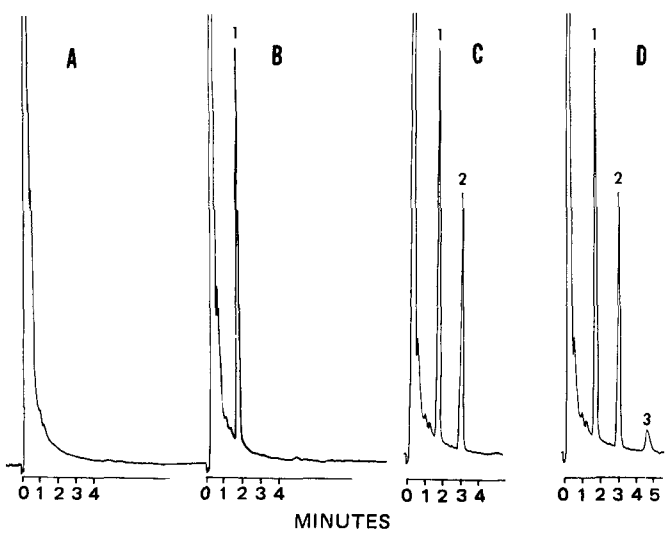

Figure 1—*GLC tracings of extracted serum samples. Key: A, serum blank; B, serum standard containing the internal standard; C, serum standard containing the internal standard and chlordiazepoxide at 0.6 μg/ml of serum; D, serum standard containing the internal standard, chlordiazepoxide, and demoxepam at 4 μg/ml of serum; peak 1, internal standard; peak 2, chlordiazepoxide; and peak 3, demoxepam.*

Table III—Assay Accuracy

Chlordiazepoxide Concentration, μg/ml		Percent Difference
Actual[a]	Calculated	
0.10	0.12	+20.0
0.25	0.21	−16.0
0.75	0.80	+6.7
1.00	1.04	+4.0
1.50	1.54	+2.7
0.62	0.60	−3.2
1.25	1.10	−12.0
0	0	0

[a] Serum spiked with chlordiazepoxide at the indicated concentrations.

Calculations—A calibration curve was constructed by plotting the peak area ratio (chlordiazepoxide to internal standard) against the serum chlordiazepoxide concentration. The unknowns were calculated using least-squares linear regression analysis.

RESULTS AND DISCUSSION

Figure 1 shows a chromatogram of an extract from 0.4 ml of serum spiked with chlordiazepoxide. Peak shapes were sharp and symmetrical. Injection of methanolic trimethylanilinium hydroxide solutions of chlordiazepoxide produced a thermal reaction in the injection port to give the methyl derivative. The flash-heater methylation technique was used previously for the barbiturates assay (5, 6).

The serum blank sample (Fig. 1) gave a very clean background on the chromatogram. The short retention time of chlordiazepoxide (R_t = 3.0 min) allows assay of many samples per day.

The precision of the assay is shown in Table I. Relative standard deviations ranged from 1.7 to 27.2%. The calculated sensitivity (Table II) was 0.045 μg/ml. The accuracy of this assay was estimated by analyzing eight spiked serum unknowns under "blind" conditions. The results (Table III) show good accuracy.

Demoxepam, a metabolite of chlordiazepoxide, also can be analyzed by this procedure. Under the same assay condition, its retention time was 4.4 min (Fig. 1) and the amount of demoxepam detectable was about 0.8 ng/injection.

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[9] Maxi-Mix model M-16715, Thermodyne, Sybron Corp.
[10] Hewlett-Packard, Avondale, Pa.
[11] Hewlett-Packard model 7670A.