Analysis of Butaclamol in Serum by Fluorescence Induction

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Abstract □ A sensitive TLC–fluorometric method was developed for the analysis of butaclamol, a benzo[6,7]cyclohepta[1,2,3-*de*]-pyrido[2,1-*a*]isoquinoline derivative, in serum. The method involves cyclohexane extraction of serum samples followed by TLC of the concentrated extracts. The developed TLC plates were sprayed with an oxidizing reagent and heated at 110°. Highly fluorescent spots were produced for butaclamol, which was well separated from metabolites and serum components. Fluorometric densitometry permitted quantitation with a sensitivity of 10 ng/spot application.

Keyphrases □ Butaclamol—TLC–fluorometric analysis, human serum □ TLC–fluorometry—analysis, butaclamol in human serum □ Fluorometry–TLC—analysis, butaclamol in human serum □ Tranquilizers—butaclamol, TLC–fluorometric analysis, human serum

Compounds of the benzo[6,7]cyclohepta[1,2,3-*de*]-pyrido[2,1-*a*]isoquinoline ring system have been prepared and shown to have potential use as neuroleptic agents (1). One important candidate was butaclamol hydrochloride (I).

Studies on orally and intravenously administered ^{14}C-butaclamol in dogs[1] receiving 1.5 mg/kg demonstrated peak serum levels of unchanged butaclamol corresponding to about 20 ng/ml. Therefore, a sensitive and specific quantitative analytical procedure for the drug in serum was required for studies in humans.

GC analysis using derivatization of the tertiary hydroxy function to the trimethylsilyl ether was useful but did not give adequate sensitivity for serum concentration determinations in the range required. Attempts to prepare derivatives suitable for electron-capture detection were unsuccessful.

UV spectrodensitometry after TLC also was unsuitable for blood level analyses because of insufficient sensitivity. Although butaclamol did not exhibit natural fluorescence, its oxidation products were highly fluorescent. Furthermore, studies indicated that oxidative induction of fluorescence could be accomplished directly on thin-layer plates.

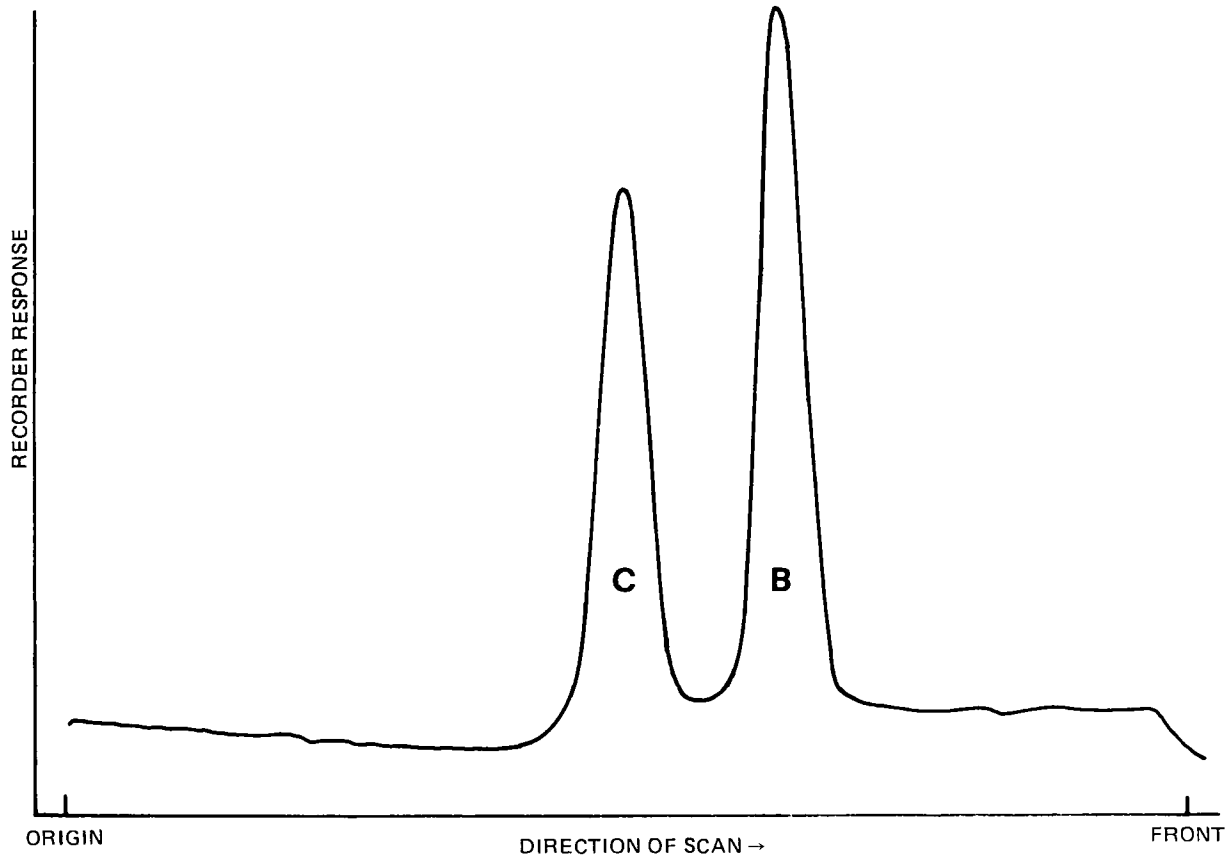


Figure 1—*Thin-layer chromatogram of butaclamol (B) and butaclamol* cis*-isomer (C).*

[1] M. Cayen, Department of Biochemistry, Ayerst Research Laboratories, Montreal, Quebec, Canada, unpublished data.

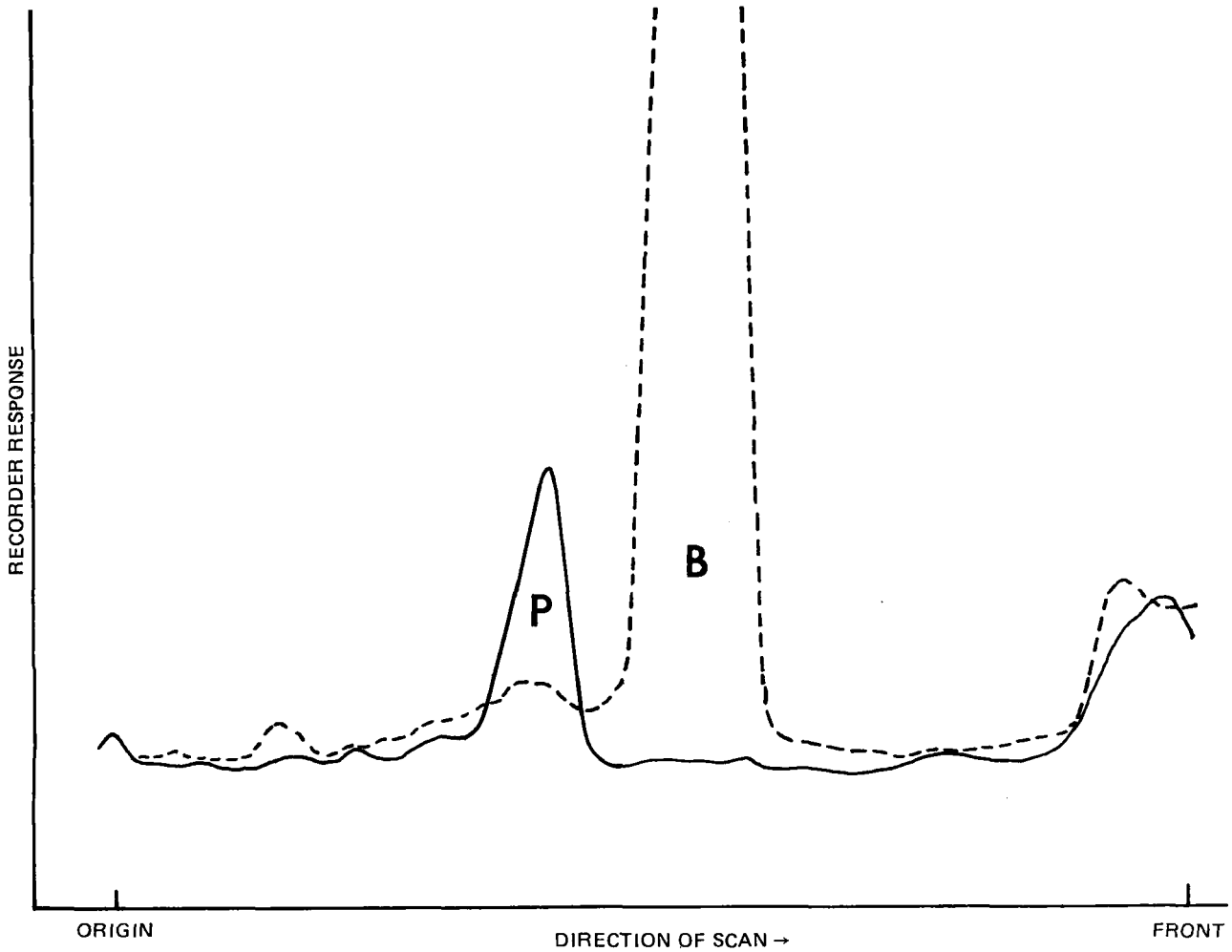


Figure 2—*Thin-layer chromatogram of butaclamol before and after reagent spraying. Key: —, before; - - -, after; P, oxidation products; and B, butaclamol.*

Although fluorescence behavior of *N*-heterocycles is not predictable, generally fluorescence can be observed where the influence of the lone electron pair of the nitrogen atom is negated in some way (2). For example, quinoline is nonfluorescent in neutral solution. However, in acid it has moderate fluorescence, presumably as a result of nitrogen atom lone pair protonation. To obtain the sensitivity needed for the anticipated serum concentration, a TLC procedure was developed to include oxidation on the plate.

Various oxidizing reagent sprays have been described (3). Several different sprays were evaluated with butaclamol, and the one giving the optimum response and stability was selected. The minimum quantifiable amount of butaclamol was 10 ng/spot; thus, this method could be used to quantitate 5 ng or less of drug/ml of serum.

EXPERIMENTAL

Reagents and Materials—TLC plates (20 × 20 cm) of silica gel[2] 60 F-254 with fluorescent indicator were used. All solvents and reagents were of analytical reagent grade, except for formic acid[3] (97%, practical) and zinc chloride[4] (dry, granular, ACS grade).

Developing Solvent System for TLC—A mixture of chloroform–ethyl acetate–ethanol–formic acid (36:36:25:3) was prepared and used in a saturated development chamber.

Fluorescence-Inducing Reagent Spray—Eighty milliliters of distilled water and 60 ml of hydrochloric acid were combined with stirring. When the solution cooled to room temperature, 20 ml of nitric acid, 1.5 g of manganese dioxide, and 1.5 g of zinc chloride were added and stirred for 30 min. The solution was then filtered with suction through a medium pore, sintered-glass funnel and transferred to a spray bottle. The solution was yellow initially but faded with time. This change had no apparent effect on the sensitivity of the spray, which was stable for at least 2 weeks.

Extraction—Five milliliters of serum and 10 ml of cyclohexane were shaken in a glass-stoppered centrifuge tube for 15 min and centrifuged; then 8 ml of the organic phase was removed to a clean tube. Ten milliliters of cyclohexane was again added to the serum, and the procedure was repeated with 10 ml of cyclohexane removed and combined with the initial extract. The combined extracts were evaporated to dryness under nitrogen at 50°[5].

To determine the extraction recovery obtained by this procedure, a known amount of the drug was spiked in human serum, extracted, and analyzed. Two extractions were required to minimize variability and to optimize recovery of butaclamol.

Chromatography and Detection—The extract residue was dissolved in 100 μl of cyclohexane by gently swirling the tube for about 30 sec. A 40-μl aliquot was taken and spotted in a prescored 1-cm TLC plate channel. Appropriate standards had been previously applied to separate channels.

The plate was developed in a saturated chamber until solvent

[2] E. Merck.
[3] Eastman Chemical.
[4] Fisher Scientific.

[5] N-Evap, Organomation Associates.

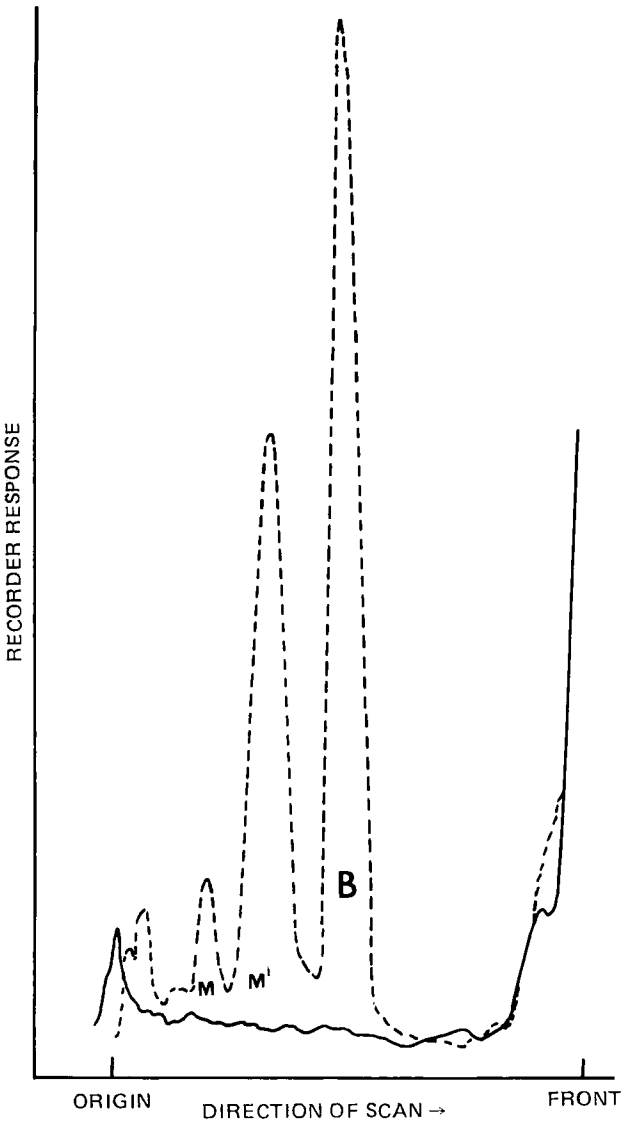


Figure 3—*Thin-layer chromatograms of serum samples from a dog where butaclamol corresponds to 54 ng/ml. Key: —, 0 hr; - - -, 2 hr; M and M', apparent metabolites; and B, butaclamol.*

front migration was about 16 cm, which took about 1 hr. The plate was then oven dried at 110° for 5 min, cooled briefly at room temperature, and finally sprayed. When saturated with spray, the plate was reheated at 110° for 30 min to induce oxidation.

Quantitation—The plates were scanned by spectrodensitometry[6] in the fluorescence mode. The excitation wavelength was 360 nm, and the emission wavelength was 710 nm. The quantitation of the unknown samples was based on a calibration curve obtained with a set of known reference samples. The observed chromatogram peak areas[7] were plotted against nanograms of reference sample applied to a given channel.

Animal Experiment—To assess the applicability of the assay method, a beagle dog, ~10 kg, was given 4 mg iv of butaclamol in 10 ml of 5% ethanol–water. At suitable time intervals before and after dosing, a 10-ml sample of blood was drawn, allowed to clot, and centrifuged. The serum was immediately frozen to await analysis.

Human Experiment—Five healthy male subjects between the ages of 18 and 55, with body weights between 45 and 90 kg and of a height proportional to size, were selected for study. A capsule dosage form containing 16 mg of butaclamol was administered, and a series of blood samples were drawn over the first 12 hr after dosing, with blank samples at zero time.

[6] Model SD3000, Schoeffel Instrument Corp.
[7] Honeywell recorder, Electronik 194, with disk integrator.

Table I—Recovery of Butaclamol from Spiked Human Serum

Concentration of Butaclamol in Spiked Sample, ng/ml	Concentration of Butaclamol Recovered, ng/ml	Recovery, % Butaclamol
10.0	9.2	92
10.0	9.2	92
10.0	9.0	90
10.0	9.5	95
10.0	10.2	102
20.0	19.4	97
20.0	19.4	97
20.0	18.8	94
20.0	17.2	86
30.0	28.1	94
30.0	27.6	92
30.0	29.1	97
30.0	27.3	91

RESULTS

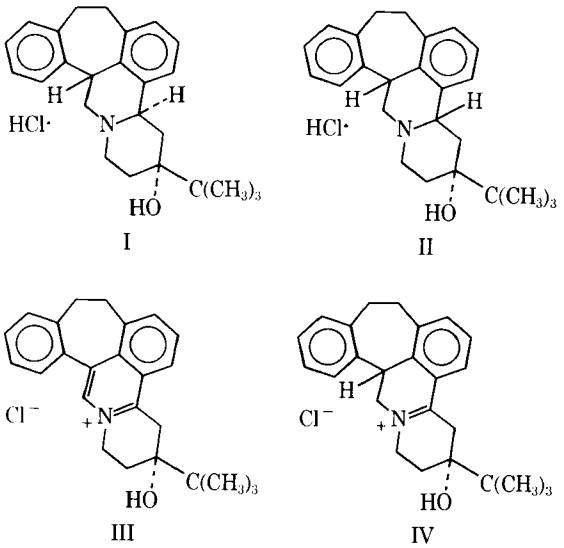
Specificity—The specificity of the TLC separation was reflected by the observed baseline separation of butaclamol (I) from its *cis*-isomer (II) (Fig. 1) and from its oxidation products (Fig. 2). These oxidation products also could be distinguished from butaclamol by their natural fluorescence, observed prior to detection with the oxidizing spray reagent.

The natural fluorescence of the oxidation products and their higher polarity (low TLC R_f) may be due to a quaternary amine structure (III and IV) and unsaturation of the heterocyclic ring. Such structures were previously prepared and identified as oxidation products of related compounds of this series[8]. Oxidation on the TLC plate caused both increases and decreases in oxidation product fluorescence but a dramatic increase in the fluorescence for butaclamol (Fig. 2).

Several oxidizing sprays including potassium permanganate, hydrogen peroxide, and nitrohydrochloric acid were evaluated. All seemed to induce fluorescence of butaclamol. However, the selected reagent spray appeared to give the highest degree of induction with the best consistency and experimental ease.

A further demonstration of the specificity of the TLC separation is shown in Fig. 3. The chromatogram of blank dog serum is compared to that obtained for a 2-hr serum sample. Clearly, butaclamol was well separated from both serum components and two apparent metabolites, M and M'.

Calibration Line, Sensitivity, and Recovery—The lowest detectable amount of butaclamol was 2 ng/spot. Since an area under



[8] L. Humber, Department of Chemistry, Ayerst Research Laboratories, Montreal, Quebec, Canada, unpublished data.

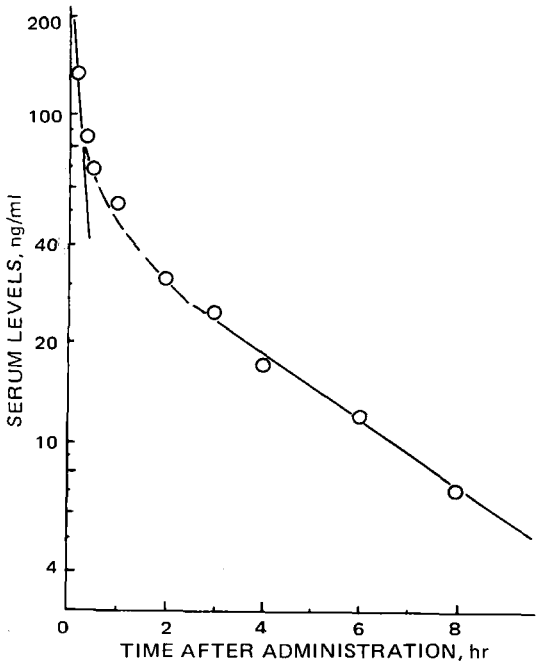


Figure 4—*Serum levels of butaclamol in a dog after intravenous administration of 4 mg. The half-life estimated for the β-phase was 3.0 hr.*

a peak of 10 relative area units can be integrated with good reproducibility, the lowest quantifiable amount was 10 ng/spot. The amount is equivalent to 5 ng/ml of serum concentration when a 40-μl sample is applied. Application of larger sample volumes would give a lower sensitivity limit. Five TLC plate spot applications ranging from 11 to 99 ng/spot gave the following calibration line: $\hat{X} = 12.50 + 3.227Y$, where $\hat{X}$ = estimated amount of butaclamol in nanograms per spot, and Y = observed relative peak area. The mean deviation from the regression line was 8.945, and the reproducibility expressed by $Syx/\bar{Y}$ was 4.71%.

A representative recovery experiment was carried out using 13 human serum samples spiked with three different known quantities of butaclamol (Table I). The mean recovery and standard deviation were 94 ± 4% with the range of 86–102%.

Serum Levels in Dog—The applicability of the assay method was tested with a dog. The serum concentration of butaclamol after administration of 4 mg iv is shown in Fig. 4. A biexponential decay with a half-life of 3.0 hr for the β-phase was observed.

Serum Levels in Humans—Mean serum butaclamol concentrations after a 16-mg oral administration in five healthy human volunteers are given in Fig. 5. A half-life for the β-phase was estimated to be 3 hr and was similar to the findings in dogs.

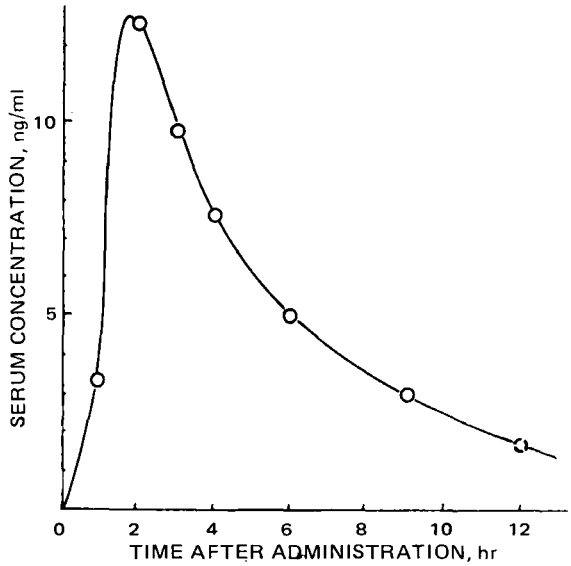


Figure 5—*Mean serum butaclamol concentration in five human volunteers after oral administration of 16 mg.*

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(3) "Thin Layer Chromatography," E. Stahl, Ed., Springer-Verlag, New York, N.Y., 1965.

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