Table III—Analysis of Oxfendazole Level in Spiked Cow Milk

Oxfendazole Spiked, μg/g	Oxfendazole Found, μg/g	Mean	Ratio of Assayed to Spiked Milk
0.005	0.0051	0.0051	1.02
	0.0050		
	0.0052		
	0.0052		
0.01	0.010	0.0105	1.05
	0.010		
	0.011		
	0.011		
0.02	0.021	0.0205	1.03
	0.020		
	0.021		
	0.020		
0.03	0.030	0.030	1.00
	0.030		
	0.030		
	0.030		
0.04	0.040	0.0405	1.01
	0.040		
	0.042		
	0.040		
0.05	0.052	0.0503	1.01
	0.049		
	0.050		
	0.050		

and the internal standard are shown in Fig. 1. The chromatogram obtained from the analysis of drug-free milk showed no interference peak at the retention time of oxfendazole and the internal standard. Chromatograms from milk spiked with 0.005 or 0.03 μg of oxfendazole/g along with 0.02 μg of the internal standard/g indicated adequate peak response for both compounds at these concentrations.

The calibration curve obtained from the analysis of blank milk spiked with oxfendazole in the 0.005–0.05-μg/g concentration range was linear. The statistical parameters showing linear regression fits of four calibration curves are given in Table II. Table III summarizes the data obtained from the repeat analysis of milk samples spiked with oxfendazole.

Coefficient of variance (standard deviation per mean) was calculated to assess assay variability. The coefficients of variance for the analysis at 0.005, 0.01, 0.02, 0.03, 0.04, and 0.05 μg/g ($n = 4$) were 1.95, 4.84, 2.94, 0.66, 1.98, and 1.99%, respectively.

Oxfendazole levels in the milk of a cow following the administration of 5 mg of oxfendazole/kg were determined by the described method. The cow was milked at 0 (predose), 4, 8, 12, and 24 hr after dose on the 1st day. For the next 6 days, milking was carried out every 12 hr. The oxfendazole levels in milk 4, 8, 12, 24, 36, 48, 60, and 72 hr following dose administration were 0.097, 0.212, 0.334, 0.460, 0.325, 0.173, 0.058, and 0.009 μg/g, respectively. Oxfendazole was not detected beyond 72 hr after dose.

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ACKNOWLEDGMENTS

Presented in part at the APhA Academy of Pharmaceutical Sciences, Washington, D.C. meeting, April 1980.

The authors thank Dr. Colin Beard and Dr. Charles Dvorak for the synthesis of the internal standard. The authors are indebted to Marian Fass for technical assistance, and thanks also are extended to Leigh Ann Wolfe for typing the manuscript.

GLC Determination of Itanoxone in Biological Fluids

HENRI COUSSE ˣ, JEAN-PAUL RIBET, and JEAN-CLAUDE VEZIN

Received August 11, 1980, from the *P. Fabre S.A. Research Center, 17 Avenue Jean Moulin, 81106 Castres, France.* Accepted for publication January 6, 1981.

Abstract □ A sensitive and reliable method for the quantitative determination of itanoxone, 4-[4′-(2-chlorophenyl)phenyl]-4-oxo-2-methylenebutanoic acid, in biological fluids is described. A quantitative ethyl acetate extraction of the plasma samples is followed by reduction and methylation of itanoxone. Quantification is achieved by GLC using electron-capture detection and an internal standard. The minimum concentration of itanoxone detected in plasma is 0.1 μg/ml. Recovery of the titrated compound added to human plasma averaged 100.9 ± 2.92% (*RSD*).

Keyphrases □ Itanoxone—GLC determination in biological fluids □ GLC determination, electron-capture detection—analysis, itanoxone, biological fluids □ 4-[4′-(2-Chlorophenyl)phenyl]-4-oxo-2-methylenebutanoic acid—itanoxone, GLC determination in biological fluids

Itanoxone (1), 4-[4′-(2-chlorophenyl)phenyl]-4-oxo-2-methylenebutanoic acid (I), is used in the treatment of metabolic disorders (2–10). The impurities likely to be found in this raw material were studied (11), as was a purification process (12). The present study perfected a sensitive and reliable method for studying the absorption, metabolism, and excretion of I in animals and humans at relatively low doses.

TLC methods were described for the determination of I in pharmaceutical formulations[1] (13). Quantitation was achieved using the UV-absorbing properties of I. The method described in this paper used GLC followed by electron-capture detection for the determination of small quantities of I in biological fluids and pharmaceutical formulations.

EXPERIMENTAL

Reagents and Materials—Itanoxone[2] (I) and internal standard (II) were obtained by a Friedel–Crafts reaction between itaconic anhydride and the corresponding aromatic derivative (14). The standard solutions

[1] A. Boucherle, University of Grenoble, Grenoble, France, expert report.

[2] F 1379.

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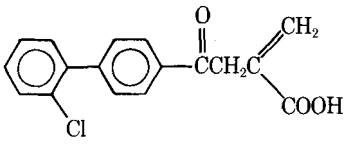
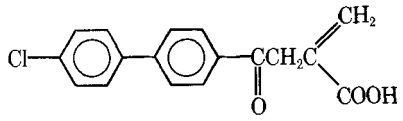
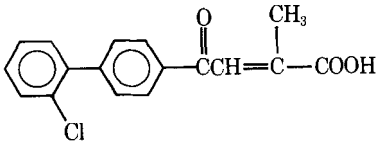


I



II



III

of the products to be analyzed were stored in glass containers at 5°. All solvents were analytical reagent grade, and the reducing catalyst was 10% palladium-on-charcoal[3].

Diazomethane (15, 16), used as the methylating agent, was obtained from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide[4] as follows. A 250-ml distilling flask was fitted with a condenser for distillation and with a funnel. The condenser was connected to two 50-ml receiver tubes immersed in ice water. Ether (10 ml) was placed in the first receiver, and 35 ml of ether was placed in the second. The outlet tube passed above the surface of the acetic acid solution in an erlenmeyer flask.

In the distilling flask was introduced 5 ml of 60% (w/w) potassium hydroxide aqueous solution, and then 5 ml of 2-ethoxyethanol and 3 ml of ether were added. In the dropping funnel was placed 20 ml of 10% (w/w) *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide in ether. The flask was heated in a water bath at 70°, and the ethereal solution was transferred gently into the flask at a regular rate during 15–20 min. The diazomethane that formed was driven off by ether and collected in the two receivers.

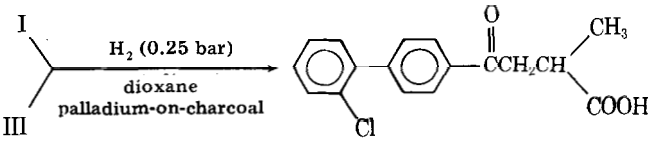
This semimicromethod does not present any serious danger of explosion or toxicity if handled carefully under a well-ventilated hood. To avoid accidental exposure to diazomethane, work was performed in a saturated acetic acid atmosphere under a hood. The ethereal solution of diazomethane should be kept in hermetically stoppered glass tubes and stored at 5° for up to 1 week.

In compliance with these safety rules, the diazomethane solution can be titrated at 0° with an excess of accurately titrated benzoic acid solution. The benzoic acid must be in excess as evidenced by the complete decolorization of the diazomethane solution. The unreacted benzoic acid is determined using 0.2 *N* alcoholic potassium hydroxide in the presence of phenolphthalein. Any diazomethane accidentally given off is neutralized immediately by acetic acid into atoxic methyl acetate.

Instrumentation—A gas chromatograph[5] was fitted with a nickel 63 electron-capture detector, an integrator[6], and a recorder[7]. A densitometer[8] and a reciprocating shaker[9] also were used.

TLC—The products obtained by synthesis or contained in pharmaceutical formulations were evaluated on thin layers (250 μm) of silica gel 60 F_{254}[3], ascendingly developed with methanol–chloroform (15:85) in a saturated atmosphere (13). Each sample was taken up in 0.1% (w/w) ethanol, and 5-μl aliquots were spotted. After development to within 10 cm of the solvent front, the plates were dried and the spots were visualized by irradiation of the plates under a UV lamp (254 nm). Under these conditions, I, II, III, and IV had R_f values of 0.42, 0.35, 0.29, and 0.47, respectively.

This technique also was used to analyze IV by fluorescence inhibition (13). Quantitative determination was carried out by comparison with a range of standards chromatographed under the same conditions as the samples. Aliquots equivalent to 1.5, 3.5, 7.5, and 12.5 μg of IV standard ethanolic solution likewise were spotted on the plate. A calibration curve



IV

Scheme I

was obtained by plotting the percentage of fluorescence inhibition *versus* known concentrations of IV in ethanol. Values for unknown concentrations of IV in samples were calculated from the standard curve.

GLC Conditions—Analyses were performed using a glass column, 1.1 m × 2 mm i.d., packed with 3% OV-225 (phenylcyanopropyl) on Chromosorb W.A.W. (80–100 mesh). The column was conditioned for 24 hr at 260° with a nitrogen flow of 10 ml/min. Gases (nitrogen, compressed air, and hydrogen) used for chromatography were purified with filters containing 4-Å molecular sieves. The detector and injector temperatures were 290 and 250°, respectively. The column was maintained isothermally at 230°. Nitrogen was the carrier gas. The flow rates for nitrogen, hydrogen, and compressed air were maintained at 50, 20, and 400 ml/min, respectively. The pulse interval for the electron-capture detector was 20 μsec. Because of the elevated temperature of the injector, high temperature septa[10] were used.

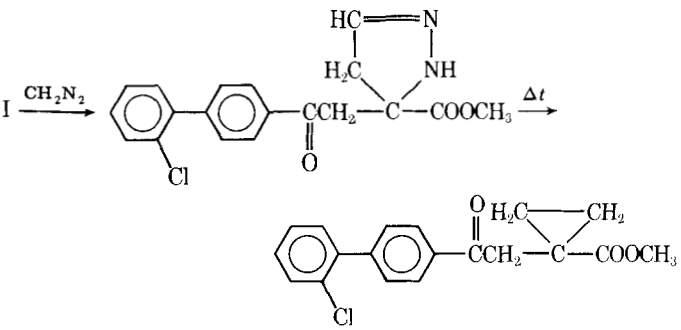
Assay—Itanoxone is a polar molecule that cannot be analyzed directly by GLC with specific columns generally used for organic acids. Compounds I and II were determined by GLC after two derivatizations, including reduction of the ethylenic bond followed by esterification of the acid group (17). Reduction was used to obtain a single compound from I and its isomer (III) which might form during extraction and chromatography at high temperature (Scheme I). Reduction also avoids the formation of a cyclopropane derivative (VI) by the addition of diazomethane to the ethylenic bond (Scheme II). Quantitation by TLC has been used at this step for I in pharmaceutical formulations and synthesis products.

Chromatography at low concentrations, such as those found in blood and urine, required an electron-capture detector for good sensitivity. By derivatizing the acid group, polarity was decreased and volatility was increased, thus providing increased sensitivity with a shorter analysis time. Methyl esters were formed quantitatively and rapidly with the diazomethane reagent.

Sample Preparation—Blood samples were collected routinely in heparinized tubes, and plasma was separated by centrifugation. Exactly 1 ml of plasma was pipetted into a 25-ml glass-stoppered extraction tube.

Plasma samples (1 ml) were spiked with 0.5 ml of the internal standard solution (10 μg). Then 1 ml of the buffer solution[11] (pH 2.2) and 10 ml of ethyl acetate were added. The preparation was shaken vigorously for 1–2 hr, and the layers were separated by centrifugation for 10 min at 3000 rpm. Ethyl acetate was transferred quantitatively to another 25-ml extraction tube and was evaporated to dryness with a gentle nitrogen stream. The residue then was dissolved in 2 ml of dioxane, and the reduction of I was performed as follows.

To the 2 ml of dioxane solution of the residue were added 0.1 ml of acetic acid in dioxane (10% w/w) and 2 mg of palladium-on-charcoal (10%). Then the solution was mixed with a magnetic stirrer; each cen-

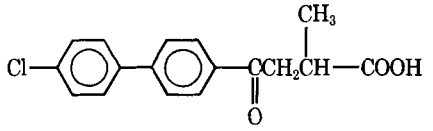


VI

Scheme II

[3] E. Merck, Darmstadt, West Germany.
[4] *N*-Methyl-*N*-tosyl nitrosamide, E. Merck, Darmstadt, West Germany.
[5] Girdel 3000, Giravions Dorand, Suresnes, France.
[6] Icap 50, L.T.T., Suresnes, France.
[7] Servotrace type PE, Sefram, Paris, France.
[8] Joyce Loebl Chromoscan.
[9] New Brunswick Scientific, Edison, N.J.

[10] Chromseptum red, Chrompack, Middelburg, The Netherlands.
[11] Consisted of sodium citrate–hydrochloric acid, E. Merck, Darmstadt, West Germany.



V

trifuge tube was stoppered and purged with nitrogen, and hydrogen was passed through it. Hydrogenation was conducted for 1 hr at 0.25 bar. After centrifugation for 10 min at 3000 rpm, the residue was extracted with 2 ml of dioxane and centrifuged. Dioxane fractions were collected in 10-ml conical glass vials, and the solvent was removed under reduced pressure (15 mm) at 60°. The residue then was dissolved in an ethereal solution of diazomethane until nitrogen bubbles disappeared. This mixture was allowed to react completely at room temperature for 1 hr. After evaporation[12], the residue was dissolved in 2 ml of hexane. Volumes of 1 μl were injected into the gas chromatograph.

Calculations—An integrator was employed for measurement of retention times and peak areas. Values for unknown concentrations of I in plasma were calculated from the internal standard (II) introduced before treatment.

RESULTS AND DISCUSSION

The TLC method was used for the quantitative determination of the reduced compound. TLC does not require expensive instrumentation, and the time required to determine the concentration of itanoxone (I) in pharmaceutical formulations varies according to the number of samples, *i.e.*, ~4 hr for six samples. Under these conditions, the minimal detectable amount is 0.5 mg/ml. A linear relationship between fluorescence inhibition and concentration is obtained for IV over the 0–15-mg/ml range. Quantification from a standard curve was adequate. To increase precision, the results are expressed on the basis of an average of three spots; reproducibility is limited to ±1%.

GLC has an advantage over previously reported methods because of its great sensitivity for I. The samples were esterified for GLC measurements. Under the assay conditions, IV and V methyl esters had retention times of 4 and 6 min, respectively (Fig. 1). The lower limit of detection sensitivity of I in human plasma extracts is 0.1 μg/ml. This value is based on a sample signal equivalent to 10% of the full-scale response. A linear relationship between detector response and concentration was obtained for I over the 0–20-μg/ml range.

Compound I was isomerized to III during GLC. The high working temperatures favor isomerization. The olefin was reduced to IV to avoid differentiation of the two isomers I and III and the side reaction with diazomethane to form a cyclopropane derivative. A series of samples containing known amounts of I was prepared to determine optimal reaction times for the formation of IV.

The results indicated that reduction of I was completed within 60 min, using palladium-on-charcoal in dioxane media (Fig. 2). Figure 3 illustrates a chromatogram of placebo human plasma extracts.

Reduction with platinum oxide as a catalyst gave small amounts of the

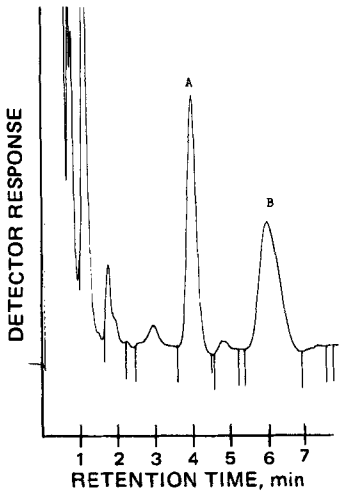
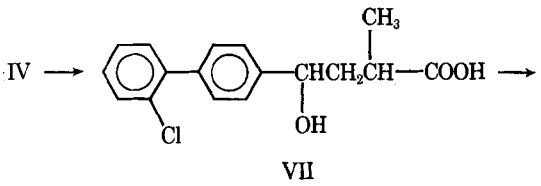
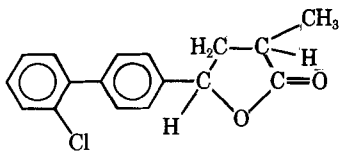


Figure 1—*Gas–liquid chromatogram (electron-capture detector) of human plasma extracts. Key: A, I reduced and esterified; and B, II reduced and esterified.*

[12] Rotavapor M, type HB 140, Buchi S. A., Flawil, Switzerland.



VII



VIII

Scheme III

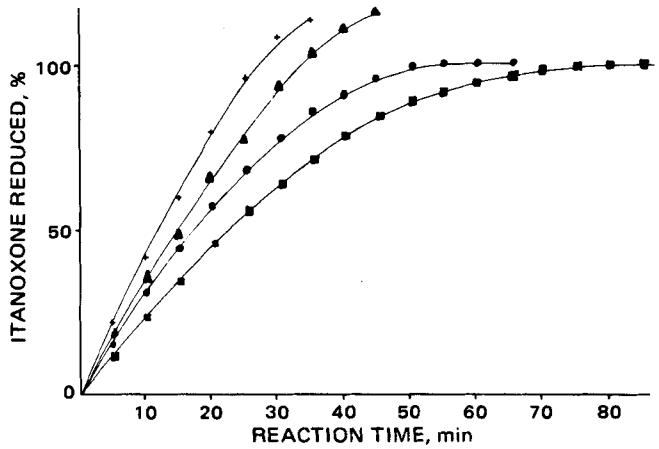


Figure 2—*Effect of reaction time and reducing medium on formation of saturated compound. Key: ●, palladium-on-charcoal in dioxane media; ▲, platinum oxide in dioxane–acetic acid; ■, palladium on aluminum oxide in dimethylformamide; and +, platinum oxide in dimethyl formamide–acetic acid media. Beyond 100% reduction, the ketonic group is reduced to a secondary alcohol.*

side-products VII and VIII. Reduction of the ketonic group occurred mainly with other catalysts such as platinum oxide, with a relative excess of catalyst and/or a high hydrogen pressure (Scheme III). Acetic acid was necessary, and up to 30% (w/w) water did not interfere with reduction.

The derivatives were stable for at least 3 weeks. The choice of II as the internal standard in the estimation of I was based on similar physicochemical characteristics. Compound II, the chemical isomer of I, was added prior to extraction. Partition coefficients of I and II were log P = 7.65 and 7.77, respectively, indicating equal extraction of both compounds.

For recovery experiments, known amounts of I and II in ethyl acetate were evaporated to dryness in glass-stoppered extraction tubes, and plasma was added. The samples were mixed thoroughly and extracted. The materials were derivatized and analyzed by GLC.

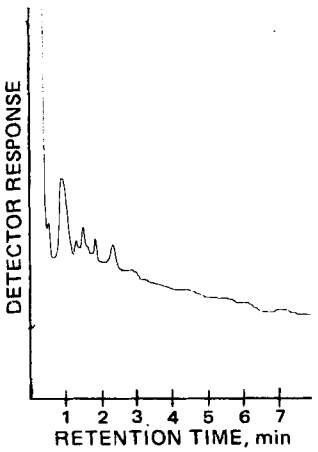


Figure 3—*Gas–liquid chromatogram (electron-capture detector) of placebo human plasma extracts.*

Table I—Accuracy and Reproducibility of GLC Analysis of I Added to Human Plasma

Added, μg/ml	Mean Recovered Amount (Range)	Recovery, %[a]
0.1	0.101 (0.097–0.103)	101
0.5	0.49 (0.480–0.51)	98
1	1.03 (0.95–1.31)	103
5	4.81 (4.74–5.33)	96.2
10	10.35 (9.84–10.72)	103.5
15	15.50 (14.68–15.96)	104

[a] Mean ± *SD* = 100.95 ± 2.92; number of analyses = 4.

The results (Table I) show the accuracy and reproducibility of the GLC analysis of I in clinical samples. The response factor was calculated using the ratio of peak areas for similar concentrations of I and II (1.22 ± 0.12). The method has been used in laboratories to determine the amount of I in rat blood compared with radioisotopic methods (18, 19). It also has been used for the determination of I in plasma and urine of humans and in pharmacokinetic studies (18).

Recently, a simple and fast high-performance liquid chromatographic method (20) was developed in which derivatization was not necessary. However, the sensitivity was not as good as that using GLC electron-capture detection.

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ACKNOWLEDGMENTS

The authors thank Mr. Gerard Amalric for technical assistance and Miss Eliane Kriner for help in translation.

Synthesis, Structure, and Features of Anilinium Anilinomethanesulfonates

MIGUEL G. NEUMANN and ROBERT A. M. C. DE GROOTE ˣ

Received September 11, 1980, from the *Instituto de Física e Química de São Carlos, USP, C.P. 369, 13560 São Carlos, SP, Brazil.* Accepted for publication January 6, 1981.

Abstract □ Two series of anilinomethanesulfonate derivatives, the anilinium and *p*-toluidinium anilinomethanesulfonates, were synthesized. They present interesting spectroscopic features and can be used as characteristic derivatives of the parent compounds. These series allow the easy study of the N–H stretching frequencies of the secondary amine whereas difficulties are encountered with the parent compounds due to the occurrence of O–H stretching bands in the same frequency range. All three compounds present a common fragmentation pattern in mass spectral analysis. Through these analyses, the salt structure of the anilinium anilinomethanesulfonates is demonstrated in opposition to a sulfonamide structure, giving further support to the salt structure of other series previously reported.

Keyphrases □ Anilinomethanesulfonates, derivatives—synthesis and structure elucidation by elemental, IR, and mass spectral analyses □ Mass spectrometry—analysis, anilinomethanesulfonate derivatives, synthesis and structure elucidation □ IR spectroscopy—analysis, anilinomethanesulfonate derivatives, synthesis and structure elucidation

Methanesulfonation of the amine groups of pharmacologically active drugs is used to increase their solubility (1) and to control their hydrolysis in biological media (2). Some common examples are *p*-phenetidinomethanesulfonate[1], dihydroergotamine[2], and dipyrone[3]. On the other hand, some researchers have studied aminomethanesulfonic compounds as potential antiviral (3) or carcinogenic agents (4, 5) since their structures are analogous to the carboxylic amino acids. As part of research into the properties of aminomethanesulfonates, the kinetics of formation of substituted anilinomethanesulfonates were studied recently (6). The inherent difficulties of analyzing IR spectra of substituted aromatic aminomethanesulfonates with regard to the O–H stretching bands also were discussed (7).

Little work has been reported concerning the analysis of the anilinomethanesulfonates, their chemical structure, and the different products that form, depending on the synthetic method. To find appropriate methods of analysis and identification of these products, a new series of derivatives was prepared by the reaction of equimolar

[1] Nevralteine.
[2] Diergotan, dihydergot, ergotex.
[3] Conmel, novalgin, sulpyrin.

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