Spectrophotofluorometric Determination of Metoserpate in Biological Systems

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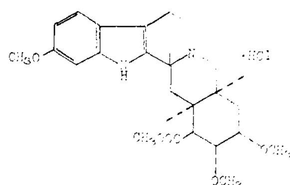
The fluorescent property of metoserpate after reaction with nitrous acid is utilized for its determination in biological systems. The procedure can easily detect 5 p.p.b. of drug. A general procedure is given for tissues (kidney, liver, muscle, and fat) which is applicable with slight modifications to eggs and blood. Average recoveries were greater than 64% in fat and greater than 74% in all other biological systems. The average controls gave an apparent metoserpate concentration of 0 p.p.b. for eggs, 2 p.p.b. for blood, while the highest average control from tissue was 2 p.p.b.

Metoserpate hydrochloride (I) (CIBA's Tradename is Pacitran) has recently received F.D.A. clearance in the veterinary field as a water soluble tranquilizer in replacement chickens.



I

To determine whether significant amounts of metoserpate hydrochloride were present in the edible portion of the chicken, it was necessary to develop methods suitable to detect 20 p.p.b. of the drug in certain tissues and 10 p.p.b. in eggs.

Haycock *et al.* (1959, 1966) have studied the induced fluorescent characteristics of reserpine under various conditions and have employed the nitrite reactions for the determination of reserpine in poultry tissue at the parts per billion level.

Use has now been made of this reaction for the determination of metoserpate in tissues, eggs, and blood (plasma). Although data are not presented for all biological systems for levels below 10 p.p.b., the procedure can easily detect less than 5 p.p.b. of metoserpate.

The method described below has been validated by the laboratories of the Food and Drug Administration, and is currently employed for regulatory purposes.

EXPERIMENTAL

Metoserpate Standard Solution. SOLUTION A. Accurately weigh 50 mg. of metoserpate and dissolve in 100 ml. of chloroform. SOLUTION B. Dilute 2 ml. of Solution A to 100 ml. with chloroform. SOLUTION C. Dilute 10 ml. of Solution B to 100 ml. with chloroform. SOLUTION D. Further dilute 10 ml. of Solution C to 100 ml. with chloroform. SOLUTION E. Finally dilute 5 ml. of Solution D with 30 ml. of chloroform and dilute to 50 ml. with methanol. Final concentration: 0.5 μg. = 50 ml. Prepare fresh daily.

ANALYTICAL PROCEDURE

Tissue. Weigh accurately about 25 grams of tissue and cut up into small pieces. Place the tissue into a Waring Blendor, previously rinsed with chloroform, and homogenize with 100 ml. of chloroform for one minute. Transfer the homogenate to a 250-ml. centrifuge bottle and centrifuge at 1500 r.p.m. for two minutes. Filter the chloroform through Whatman 2V filter paper into a 1-liter round bottom flask retaining the tissue in the centrifuge bottle. (For fat and skin, go directly from the Blendor to the filter paper.) Rinse the Blendor with a 100 ml. portion of chloroform, and transfer to the centrifuge bottle. Shake vigorously for one minute, centrifuge and filter into the round bottle flask. Repeat this washing step with an additional 100-ml. portion of chloroform. Finally rinse the filter paper with two 50-ml. portions of chloroform. Evaporate the chloroform under vacuum to about 10 ml. on a rotating evaporator at a temperature not exceeding 40° C.

Transfer the contents of the flask to a 250-ml. centrifuge bottle using, in order, 50 ml. of isooctane, 5 ml. of chloroform, and two additional 50-ml. portions of isooctane. Extract this solution with three 20-ml. portions of 2% citric acid, shaking each extract for two minutes. Centrifuge and draw off with the aid of a syringe equipped with a round-nosed needle, the citric acid solutions, and filter through Whatman No. 2V filter paper into another 250-ml. centrifuge bottle. Add 3 ml. of 10*N* sodium hydroxide to the citric acid solution and extract with three 10-ml. portions of chloroform shaking each extract gently for one minute. Centrifuge and draw off with the aid of a syringe the chloroform extracts in a 40-ml. centrifuge tube. To the chloroform extracts, add 10 ml. of 1% sodium bicarbonate, shake vigorously for one minute, and then centrifuge. Draw off the chloroform layer with the aid of a syringe and filter through cotton into a 50-ml. volumetric flask. Rinse the bicarbonate wash with 5 ml. of chloroform, centrifuge, and transfer in the same manner into the 50-ml. volumetric flask. Dilute to volume with methanol and mix well. (Sample Solution)

Fluorescence Development. Pipet duplicate 15-ml. aliquots of standard Solution E and the sample solution into separate 25-ml. volumetric flasks. To one of each, which constitute the respective blanks, add 1 ml. of 50% aqueous methanol. To the others, add 1 ml. of 0.1% sodium nitrite in 50% aqueous methanol. To all, add 0.5 ml. of hydrochloric acid, mix well, wait 30 minutes, and dilute to volume with methanol. Determine the fluorescence of all solutions on an Aminco-Bowman Spectrophotofluorometer or other suitable instrument, setting the instrument so that the standard gives a reading of approximately 80.

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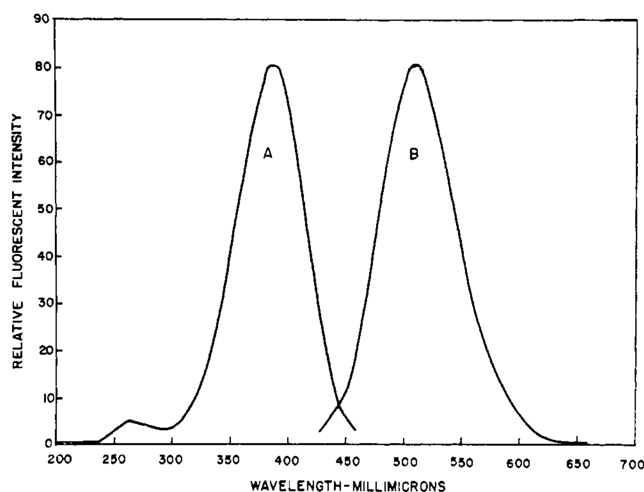

Figure 1. Fluorescent excitation and emission spectra of metoserpate

A. Excitation spectrum at emission of 510 mμ
B. Emission spectrum at excitation of 390 mμ

Table I. Recovery of Metoserpate from Biological Systems

System	P.P.B. Added	P.P.B. Found[a]	Mean Recovery, %
Muscle	0	1 ± 0.2 (5)[b]	...
	20	16.1 ± 1.0 (9)	81
	50	38.7 ± 2.0 (3)	77
	100	77.0 ± 3.0 (3)	77
Fat (Skin)	0	2 ± 0.2 (5)	...
	20	12.7 ± 0.9 (6)	64
	50	37.4 ± 0.5 (3)	75
	100	77.8 ± 4.1 (4)	78
Liver	0	2 ± 0.1 (5)	...
	20	14.7 ± 0.7 (6)	74
	50	38.6 ± 1.8 (3)	77
	100	90.5 ± 1.0 (2)	91
Kidney	0	1 ± 0 (5)	...
	20	16.6 ± 0.2 (5)	83
	50	45.4 ± 0 (2)	91
	100	98.7 ± 5.0 (3)	99
Eggs	0	0 ± 0 (2)	...
	5	4.9 ± 0.1 (5)	98
	10	10.0 ± 0 (3)	100
	20	17.6 ± 0.1 (4)	88
Blood (Plasma)	0	2 ± 0.1 (2)	...
	20	20.0 ± 0.2 (3)	100
	60	60.3 ± 0.3 (3)	100
	100	104.0 ± 1.0 (4)	104

[a] All recoveries corrected for their respective control.
[b] Numbers in parentheses represent the number of analyses run.

Calculation:

$$\frac{F_s - F_{sB}}{F_{std} - F_{stdB}} \times \frac{500}{C} = \text{p.p.b. metoserpate} \quad (1)$$

F_s = Fluorescence of sample
F_{sB} = Fluorescence of sample blank
F_{std} = Fluorescence of standard
F_{stdB} = Fluorescence of standard blank
C = Weight of sample

For the Aminco-Bowman Spectrophotofluorometer the instrument settings are as follows: activation wavelength, 390 mμ ± 10 mμ; fluorescence wavelength, 510 mμ ± 10 mμ; slit arrangement, 3 mm, 2 mm, --, --, 2 mm, 3 mm, 3 mm; and photomultiplier tube, R.C.A. 1P 21.

Eggs. Weigh the contents of one egg and transfer to a 250-ml. centrifuge bottle. Add 125 ml. of chloroform, stopper, and shake vigorously for three minutes. Centrifuge for five minutes at 1500 r.p.m., withdraw the chloroform with the aid of a syringe, and transfer to a 1-liter round bottom flask, filtering the chloroform through Whatman No. 2V filter paper. Repeat the extraction with a 50-, 75-, and 100-ml. portion of chloroform, combining all chloroform extracts in the 1-liter flask. Evaporate the chloroform to about 5 ml., under vacuum, on a rotating evaporator at a temperature not exceeding 40° C. Transfer the contents of the flask to a 250-ml. centrifuge bottle using, in order, 40 ml. of *n*-hexane, 5 ml. of chloroform and two 50-ml. portions of *n*-hexane. Proceed as under Tissue beginning with "extract this solution with three 20-ml. portions of 2% citric acid..." The calculation is the same as in Equation 1 except C = weight of egg instead of tissue.

Blood (Plasma). Centrifuge the blood at 1500 r.p.m. for several minutes and pipet 5 ml. of the plasma into a 40-ml. centrifuge tube. Add 5 ml. of 3*N* HCl and mix well. Extract with three 10-ml. portions of chloroform, transferring the chloroform extracts with the aid of a syringe to another 40-ml. centrifuge tube. Wash the combined chloroform extracts with 10 ml. of 1% sodium bicarbonate and filter the chloroform through cotton into a 50-ml. volumetric flask, drawing off the chloroform with the aid of a syringe. Wash the sodium bicarbonate with 5 ml. of chloroform and add the chloroform to the previous extracts. Dilute to volume with methanol and mix well. For fluorescence development, proceed as directed under Tissue. The calculation is the same as in Equation 1 except C = ml of plasma used instead of tissue.

RESULTS AND DISCUSSION

The fluorometric nitrite procedure utilized by Haycock *et al.* (1959, 1966) for the determination of reserpine and other rauwolfia alkaloids has been adapted for the determination of metoserpate in tissues, eggs, and blood (plasma). Figure 1 shows the fluorescent excitation and emission spectra of metoserpate under conditions of the assay. The procedure has been validated by two FDA laboratories and will be used in the future for regulatory purposes.

Table I shows the recovery of metoserpate from various biological systems at the 20-, 50-, and 100-p.p.b. levels. Except for fat (skin) at the 20-p.p.b. level, where the average recovery was 64%, recoveries from all other systems averaged 74% or better. The highest average control from the various biological systems gave an apparent metoserpate concentration of 2 p.p.b.

All recovery data shown in Table I have been corrected for control values. Since unreacted (nitrite free) samples containing metoserpate gave fluorescent readings which were similar to actual nitrite reacted controls, the necessity of assaying controls is eliminated, and a portion of the sample solution can then act as the control blank.

Although no data are given for metoserpate below 20 p.p.b. in tissues and plasma, the procedure can easily detect 5 p.p.b. in these systems. Metoserpate has been found to be linear up to at least 0.0125 μg./ml. of final solution which well covers the range where any levels may be seen.

LITERATURE CITED

Haycock, R. P., Sheth, P. B., Connolly, R. J., Mader, W. J., J. Agr. Food Chem. **14**, 437 (1966).
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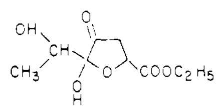
Correction

BIOLOGICALLY ACTIVE COMPOUNDS FROM FIELD FUNGI

In this article by P. M. Scott and Emmanuel Somers [J. Agr. Food Chem. **17,** 430 (1969)], the structural formulas, identified by Roman numerals in the text, were omitted. They are as follows.



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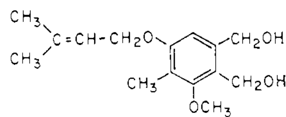

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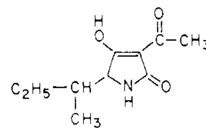

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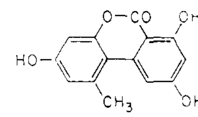

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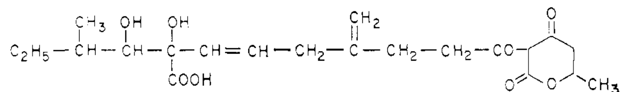

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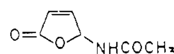

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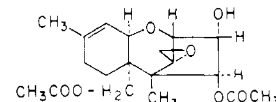

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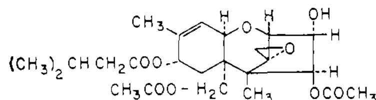

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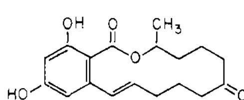

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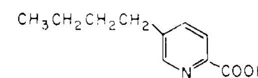

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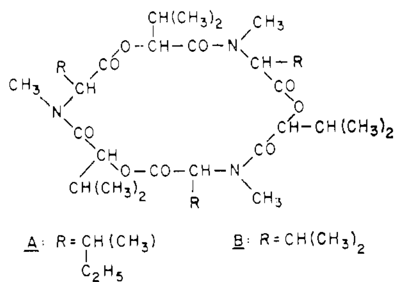

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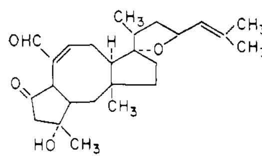

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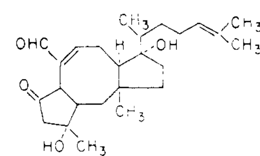

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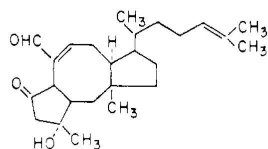

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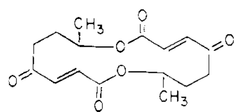

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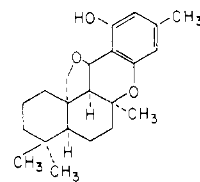

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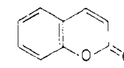

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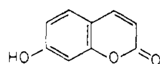

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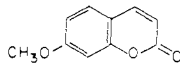

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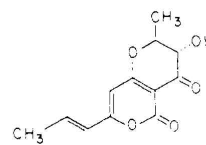

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