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Note

Thin-layer chromatographic test for reserpine in plasma

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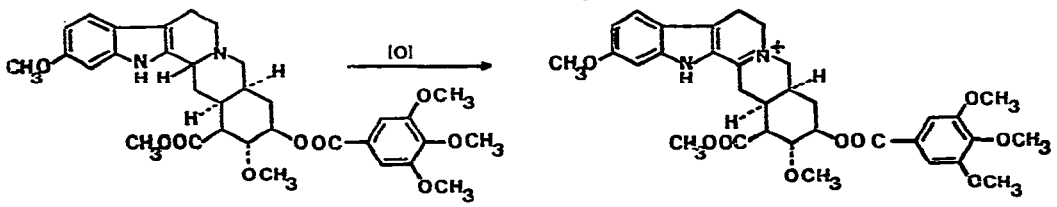
Reserpine (I), the principal alkaloid of *Rauwolfia serpentina* (Benth), depletes stores of catecholamines and 5-hydroxytryptamine in the brain and other tissues[1]. Consequently, reserpine has been used clinically as an antipsychotic and an antihypertensive agent. Because it produces a state of indifference to environmental stimuli, it has allegedly been administered to horses prior to participation in shows, training events, dressage competitions, and exhibitions. The American Horse Shows Association, among others, specifically prohibits its use in such events but has been unable to enforce this regulation because of the difficulty of detecting reserpine in either blood or urine samples collected from horses. Numerous attempts to develop a sensitive and specific method for detecting reserpine in horses have been made, but no method has yet proved satisfactory.



I II

It has been shown that reserpine solutions develop fluorescence under a variety of conditions including exposure to light[2,3], and treatment with acids[4] or oxidizing agents[5,6]. Several products may be produced but the predominant product and the one exhibiting the greatest fluorescence[6] is 3-dehydroreserpine (II).

Haycock *et al.*[7] showed that the excitation and fluorescence maxima of 3-dehydroreserpine are 390 and 510 nm, respectively. The fluorescent intensity at 510 nm is more than 12 times the fluorescent intensity of reserpine at 360 nm following excitation at 280 nm. Utilizing this fluorescence enhancement, Tripp *et al.*[8] developed a thin-layer chromatographic procedure for quantitation of reserpine in human plasma following oral and intramuscular administration of single, total doses of 1

mg of reserpine. They measured sub-nanogram quantities of reserpine by *in situ* densitometry following conversion of reserpine to 3-dehydroreserpine by treatment with acetic acid vapors. In our opinion, this method appeared to possess sufficient sensitivity and specificity to justify evaluation as a method for detecting reserpine in horses. We, therefore, evaluated and subsequently modified this procedure for routine analysis of plasma samples for the presence of reserpine.

EXPERIMENTAL

Materials

High-performance thin-layer chromatography plates (silica gel 60, 10 × 10 cm, without fluorescent indicator, EM Laboratories, Inc., Elmsford, N.Y., U.S.A.) were used as obtained. All solvents were reagent grade or better and reserpine was a gift of Ciga-Geigy (Summit, N.J., U.S.A.).

A stock solution containing 100 mg of reserpine per liter of methanol was prepared. This solution, stored in the dark at 4°, was stable for at least one month. From this solution, an aqueous reserpine standard was prepared daily at a concentration of 10 μg/l.

Sample preparation

Two ml of a saturated solution of sodium tetraborate in water and 4 ml of benzene were added to 1–4 ml of equine plasma contained in glass culture tubes (16 × 125 mm). Samples were then mixed for 5 min on a Rotorack® (Model 343; Fisher Scientific Company, Springfield, N.J., U.S.A.) and centrifuged for 5 min or until the layers separated. The benzene layers were transferred to clean, dry tubes and evaporated to dryness under a stream of nitrogen in a water bath at 50°.

Thin-layer chromatography

Each dried extract was dissolved in 6 μl of dichloromethane. A 2-μl aliquot was carefully spotted, using a microliter syringe (No. 701, Hamilton, Reno, Nev., U.S.A.), one cm from the end of a thin-layer plate. Care was taken to keep the diameter of the sample spot less than 2 mm. Extracts of a known blank, 4–15 test samples, and a spiked plasma sample containing 2 μg of reserpine per liter of plasma were spotted on each 10 × 10 cm plate. Samples were then developed 5 cm in a 30 cm × 15 cm × 25 cm glass developing tank lined with filter paper and containing 100 ml of developing solvent. At the end of development, the plates were removed from the tank, briefly dried in a stream of ambient air, and placed in a glass developing tank containing a 25-ml beaker filled with glacial acetic acid. After one hour or more, plates were removed from the acetic acid chamber and examined under long-wavelength ultraviolet light (Chromatovue Model CC-20; Ultraviolet Products, San Gabriel, Calif., U.S.A.). The presence of reserpine was indicated by bright blue fluorescence at the appropriate R_F.

RESULTS AND DISCUSSION

Using the procedure described by Tripp *et al.*[8], we were unable to achieve adequate sensitivity of detection of reserpine to permit its detection in horse plasma

following 1–2-mg intramuscular doses. However, certain modifications of their procedure have made detection of reserpine in horse plasma possible.

Extraction of blank plasma samples from non-medicated horses with dichloromethane and subsequent thin-layer chromatography resulted in high background fluorescence at the R_F of reserpine thus preventing its detection in plasma extracts of medicated horses. By extracting plasma samples with benzene this high background fluorescence was almost completely eliminated while the high recovery of reserpine was maintained.

The use of high-performance thin-layer plates greatly improved our ability to detect reserpine in plasma samples. On conventional plates, the minimum detectable quantity was about 10 ng when development was carried out to 15 cm. Even when development on these plates was reduced to 5 cm, resolution was poorer and the minimum detectable quantity was higher than on the high-performance plates.

The ability to detect sub-nanogram quantities of reserpine is highly dependent on the quality of the chromatographic process. On high-performance plates this is dependent on short development (5 cm) and restriction of the diameter of the initial spot to 2 mm or less.

Reserpine had an R_F value of 0.15 when chromatographed in the chloroform–acetone solvent system described by Tripp *et al.*[8]. Several other solvent systems (see Table I) were developed in order to obtain more favorable R_F values and to provide alternative systems for confirmation of suspect samples. The chloroform–methanol system was used routinely for screening purposes since the R_F was favorable and development times were short. When sample spots with R_F value the same as authentic reserpine were observed in this system, samples were re-chromatographed in other solvent systems or confirmation.

TABLE I

R_F VALUES FOR RESERPINE IN VARIOUS SOLVENT SYSTEMS

Solvent system	R_F
Chloroform–acetone (70:30)	0.15
Chloroform–methanol (95:5)	0.50
Benzene–methanol (80:20)	0.64
Butanol–water–acetic acid (80:20:20)	0.72

If the chromatographic plates were examined before acetic acid treatment, reserpine spots could not be detected. Within an hour after initiation of acetic acid treatment, however, reserpine spots were clearly visible and maximum fluorescence developed within 24 h. The fluorescence was stable as long as the plate was stored in the acetic acid chamber and was protected from strong light. A slight blue fluorescent spot near the R_F of reserpine could be detected in all samples before acetic acid treatment. However, this fluorescence disappeared as the reserpine fluorescence developed. Thus the requirements that an extract of a test sample have a blue fluorescent spot at the same R_F as reserpine in four solvent systems and that the development of fluorescence parallel that of authentic reserpine increases the specificity of the method.

Although exposure of developed chromatographic plates to acetic acid vapors

was an effective method for producing the reserpine fluorophor, the time required for development of fluorescence was too long for rapid analysis of plasma extracts. Noting that Jakovljevic *et al.*[4] reported a sensitive fluorimetric method for determining reserpine in pharmaceutical preparations in which they heated solutions of reserpine in glacial acetic acid containing *p*-toluenesulfonic acid, we sprayed plates developed in the chloroform–methanol solvent system with a 1% solution of *p*-toluenesulfonic acid in glacial acetic acid. The fluorophor formed immediately and possessed the same fluorescent intensity as was obtained by prolonged exposure of the plates to acetic acid vapors. Currently we are using this spray to produce the fluorophor and are storing the plates in an acetic acid vapor chamber to preserve fluorescence. Other methods including exposure of the developed plates to (1) concentrated hydrochloric acid, (2) formic acid, or (3) aqua regia vapors as well as overspraying with (4) 6 *M* nitric acid, (5) ferric chloride in sulfuric acid, or (6) 1% aqueous hydrogen peroxide either failed to produce the fluorophor or were no better than exposure to acetic acid vapors.

TABLE II

RESULTS OF TESTS FOR RESERPINE IN MEDICATED HORSES

−, Reserpine not detected; +, reserpine detected; NS, no sample collected.

Horse	*Dose (mg)*	*Time (h)*									
		Pre	*1*	*2*	*4*	*6*	*24*	*48*	*72*	*120*	*144*
Gelding	1.0	−	+	+	+	+	+	NS	NS	NS	NS
Mare	1.0	−	+	+	+	+	+	NS	NS	NS	NS
Mare	1.0	−	+	+	+	+	+	NS	NS	NS	NS
Gelding	1.5	−	+	+	+	+	+	+	NS	NS	NS
Mare	2.0	−	+	+	+	+	+	+	+	+	−

In order to assess our ability to detect administered reserpine, total, single, intramuscular doses of 1.0, 1.3, and 2.0 mg of reserpine were given to 5 horses. Blood samples were collected at various times after administration and were screened for the presence of reserpine. The horses exhibited no obvious clinical response to these doses. Table II gives the results of the tests. Reserpine was detected in all samples collected after drug administration except in the 144-h sample from the single horse which received a 2-mg dose. In analyzing this sample, we tested a 4-ml aliquot of plasma and were unable to detect reserpine. Urine samples obtained from the horse administered 1.5 mg were screened for reserpine according to this procedure and were found negative for the presence of reserpine at all sample times. In order to investigate the possibility of false positive findings, twenty horses receiving various medications other than reserpine were selected at random from the Ohio State University Veterinary Hospital. Plasma samples were obtained and analyzed as described above. No false positives were observed.

Thus, we have developed a sensitive and specific procedure for detecting and confirming the presence of reserpine in equine plasma following administration of reserpine. Reserpine has been detected for as long as 120 h following a 2-mg intramuscular dose, and no false positives have been observed. The method is, therefore, suitable for testing samples collected from horses for the presence of reserpine.

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