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Comparison of UV and Fluorescence Spectrophotometry for the Quantification of a Potent Myotonia Inducer: Anthracene-9-carboxylic Acid, in Plasma, Urine, and Saline Perfusion Fluids

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Abstract □ UV and fluorescence spectrophotometry were used to establish the analytical profile of a potent myotonia inducer, anthracene-9-carboxylic acid (I). UV spectrophotometry is useful for the determination of I when it is dissolved in physiological solutions (Ringer's, Tyrode's, *etc*). In these fluids there is a linear relationship between UV absorption and I concentration between 500 and 2000 ng/ml ($2.25–9.0 \times 10^{-6}$ *M*). However, in biological fluids there are interferences in the UV absorption due to organic substances. On the other hand, fluorescence spectrophotometry is more sensitive than UV for determinations in plasma and urine. Within the range of 200–1000 ng/ml ($0.9–4.5 \times 10^{-6}$ *M*) fluorescence intensity increases linearly with concentration. Furthermore, when both emission and excitation spectra are combined there are no interferences due to organic substances normally present in those fluids. An extraction procedure of I from plasma and urine is also described, and the importance of I determinations in relation to the problem of this myotonia-inducing aromatic monocarboxylic acid is discussed.

Keyphrases □ Fluorescence spectrophotometry—comparison of UV for quantification of a potent myotonia inducer

Several classes of chemical agents can produce, both *in vivo* and *in vitro*, changes in the function of mammalian skeletal muscle resembling the condition known as myotonia congenita in humans and in goats (1, 2). These agents include the veratrum alkaloids (3), the substitution of chloride ion by other ions that do not cross the muscle membrane (4, 5), a group of hypocholesterolemic substances (6, 7), clofibric acid that reduces the triglyceride levels (8), and other aromatic monocarboxylic acids (9–11). All these substances act directly on the membrane of mammalian skeletal muscle. However, an increasing number of studies in recent years (12–14) have confirmed the early proposal of Bryant and Morales-Aguilera (15) that aromatic monocarboxylic acids induce in mammalian muscle fibers a state that more closely resembles naturally occurring myotonia congenita than do the other agents. Bryant and Morales-Aguilera (15) and Palade and Barchi (16) showed that anthracene-9-carboxylic acid (I) is the most potent inducer of myotonia among the tested aromatic monocarboxylic acids and also that these substances very specifically block the chloride channel. The availability of chemical agents whose effects resemble the naturally occurring condition is important in the development of animal models that can give information as to the mechanisms of the disease. With the exception of clofibric acid (17), there is a lack of quantitative studies regarding the *in vivo* kinetics of the myotonia-inducing aromatic monocarboxylic acids. Also, methods are not available for their chemical determination in biological fluids. In this paper we report UV and fluorescence spectrophotometric methods for I determination and compare them in blood, plasma, urine, and saline perfusates, covering most of the present analytical needs of biomedical researchers interested in the study of myotonia induced by I.

EXPERIMENTAL

Determination of I by UV Spectrophotometry—*Sodium Bicarbonate Buffer*—Fifty milligrams of I[1] and 1.5 g of sodium bicarbonate were placed in a 100-ml volumetric flask and brought to volume with water. Since I is almost insoluble in water (the concentration of a saturated water solution is 1.88×10^{-4} *M*), it was necessary to alkalinize the medium with sodium hydroxide to pH 8–9 (22°)[2] and stir vigorously for at least 20 min. This solution has 320 mosm[3]. By successive dilutions of the isotonic alkaline solution of I the following concentrations were obtained: 0.5, 1.0, 1.5, and 2.0 μg/ml. The absorbance of these solutions was measured relative to an isotonic solution blank at 255 nm[4]. These solutions are unstable in light, so it is necessary to proceed immediately; the solutions must be protected from light.

Chloroform—A 50-mg volume of I was placed in a 100-ml volumetric flask, and brought to volume with chloroform. The dilutions to 0.5, 1.0, 1.5, and 2.0 μg/ml were made with the same solvent. Their absorbance was measured *versus* a chloroform blank at 255 nm.

Excitation/Emission Spectrum of I in Chloroform—In a 50-ml volumetric flask, 25 mg of I was added, brought to volume with chloroform, and diluted to a final concentration of 10 μg/ml. The spectra of excitation and emission were obtained in a spectrophotofluorometer[5], and the results are shown in Fig. 1. The excitation spectrum exhibits a clear absorption peak at 355 nm. This wavelength was used subsequently for excitation of the samples. The fluorescence spectrum manifests an enhanced emission at 470 nm, and the peak at this wavelength was used to quantify I in blood, plasma, and urine.

[1] Aldrich Chemical Co.
[2] Coleman Instruments Model 37-A Ph Meter.
[3] Wescor Model 5100 Osmometer.
[4] Unicam Sp 800 Spectrophotometer.
[5] Aminco-Bowman.

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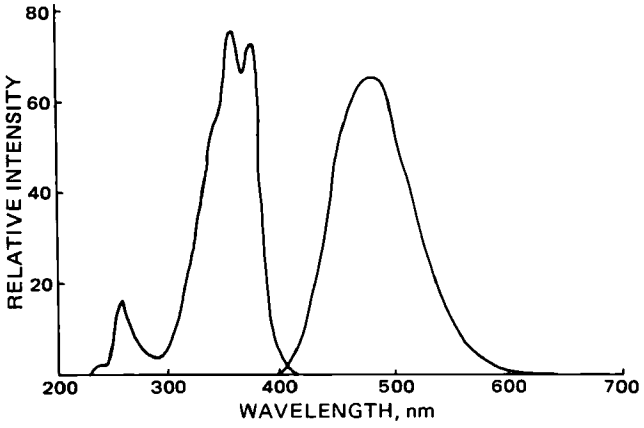

Figure 1—*Wavelength maxima for excitation (left) and emission (right) of I in chloroform (10 μg/ml).*

Standard Curve—Twenty-five milligrams of I was dissolved in chloroform and diluted to 50 ml in a volumetric flask, and then diluted with chloroform to the following concentrations: 0.2, 0.4, 0.6, 0.8, 1.0, and 1.25 μg/ml. The emission spectrum was determined for each dilution, and the intensity of fluorescence was plotted *versus* I concentration.

Spectrophotofluorometric Quantification of I in Blood, Plasma, and Urine of Rabbits—*Stock Standard*—Fifty milligrams of I and 0.75 g of sodium bicarbonate were dissolved in water and adjusted to 50 ml in a volumetric flask. This solution had 320 mosm and the pH was 8.5.

Blood, Plasma, and Urine Samples—With a syringe containing an anticoagulant[6] 10 ml of blood was drawn from the marginal vein of the ear from each of nine healthy New Zealand White rabbits. Plasma was obtained from 5 ml of this blood by centrifugation at 2000 rpm during 10 min[7]. The urine of the rabbits was collected by a catheter placed in the urinary bladder[8] and diluted 10-fold with water before analyses.

Sample Preparation—Two milliliters of whole blood were pipetted into a 15-ml centrifuge tube and mixed with 2 ml of stock standard previously diluted 10-fold. The plasma and urine were prepared in the same way.

Extraction Procedure—To 1.0 ml of blood, plasma, or urine prepared sample was added 0.5 ml of 3 *M* HCl, 0.5 g of sodium chloride, and 50 ml of chloroform in a Erlenmeyer flask. The contents were ultrasonicated[9] for 15 min, then centrifuged (full speed for about 10 min) to separate the immiscible liquid. The water layer was removed and the fluorescence of I was determinated in chloroform. The reproducibility of the assay was assessed by calculating the standard deviation of the mean value of the individual determinations.

RESULTS AND DISCUSSION

Solubility of I in chloroform is greater than in methanol or water. Therefore it is easier to work with chloroformic solutions since the extraction of I from biological fluids is faster and more efficient with this solvent.

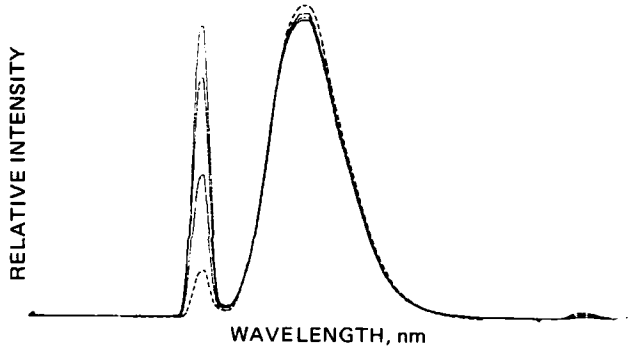

Figure 2—*Emission spectra of chloroform-extracted I from a plasma sample (n = 3). The dotted line corresponds to the standard.*

[6] Heparin, Sigma Chemical Co.
[7] Phillips-Drucker, Model L-7011 Centrifuge.
[8] Foley catheter, No. 8.
[9] Ultrasonic cleaner, Tank Unit.

Table I—Percent of Recovery of Added I [a]

Sample	Urine	Plasma	Whole Blood
1	98.0	96.0	59.0
2	100.0	96.6	60.6
3	101.2	97.7	52.1
4	101.3	98.4	72.2
5	102.0	98.4	74.1
6	101.3	100.7	76.8
7	100.5	97.8	61.3
8	105.0	102.1	63.3
9	105.0	102.1	63.3
Mean ± *SD*	101.0 ± 2	98.0 ± 2	66.5 ± 6

[a] To each of nine samples there was added 50.0 μg of I/ml. Standard curve for I using 1.0 μg/ml as a reference.

UV Spectrophotometry—Concentrations of I in sodium bicarbonate buffer and chloroform were determined by measuring the increase in absorbance at 255 nm. With the procedure for measuring I by UV detection, the peak height and the amount of I in the standards as well as in the sample are linearly related to concentration in the two solvents. The absorbance is directly proportional to I concentration of 0.5 to 2.0 μg/ml ($r^2 = 0.99$). The use of UV spectrophotometry for the determination of I is convenient when saline-perfused preparations are used because the composition of the saline solutions (Ringer's, Tyrode's, Locke's, *etc*) does not interfere with the readings. The detection limits of this method are 0.5–2.0 μg/ml ($2.25–9.0 \times 10^{-6}$ *M*). This range is within the one found in saline-perfused preparations (10^{-6}–10^{-4} *M*). In other words, in those preparations it is possible to quantify directly or after one dilution the I concentration by UV detection. On the other hand, in the case of chloroform extracts of normal blood, plasma, and urine, some constituents of those fluids gave large interfering peaks near, or at the point of maximal I absorbance in UV, interfering with its quantification. Furthermore, urine is often contaminated with blood.

Spectrophotofluorometric Determination of I—Excitation and emission spectra of I in chloroform were established because these data were not found in the literature (Fig. 1). The fluorescence spectrum obtained by this laboratory is similar to the one reported by Werner and Hercules in ethanol, although the absorption spectrum is different (18). There is a linear relationship over the range of 0.2–1.0 μg/ml, whereas higher concentrations are off because of self-quenching (19).

Compound Recovery—Table I shows the quantitative results obtained by spectrophotofluorometry for blood, plasma, and urine samples obtained from nine rabbits. The percent recovery of I added to samples and extracted with chloroform was in whole blood, plasma, and urine, 66.5 ± 7%, 98.0 ± 1%, and 101.0 ± 2%, respectively. Figure 2 shows the spectrum of a sample of plasma analyzed in triplicate. Similar results were obtained with urine (Table I).

UV Spectrophotometric *versus* Spectrophotofluorometric Assay—The sensitivity of these two formes of detection of I in chloroform were compared. The spectrophotofluorometric detection limit was found to be 200 ng/ml. The spectrophotometric UV limit was 500 ng/ml. Thus, the fluorometric method is at least twice as sensitive.

The recovery of I from plasma and urine averages 98.0 ± 1% and 101.0 ± 2%, respectively. These results indicate that the fluorometric method for plasma and urine is precise at low concentrations (Table I). However, the fluorometric method is not satisfactory for whole blood because recovery of added purified I averages only 66.5 ± 7% for whole blood.

Sensitivity—The detection limit of the fluorometric method lies in the nanogram range. This sensitivity is satisfactory because lower concentrations of I do not affect biological preparations *in vitro* (14, 15). *In vivo* a 8.0-mg/kg dose of I administered intraperitoneally to rats or intravenously to goats or rabbits elicits a clear myotonic state as well as "percussion myotonia" and the "warm-up" phenomenon. Pharmacokinetic studies in rabbits (20) shows that the same dose (8 mg/kg) produces at 1 and 120 min plasma concentrations of 1.63×10^{-4} and 1.0×10^{-5} *M*, respectively. These concentrations are similar to the ones found as active *in vitro*, and can be determined by the fluorometric method. Furthermore, the emission spectra of the chloroformic extracts of plasma and urine are clean (Fig. 2).

Specificity—To rule out interferences from other substances present in plasma or urine, we performed determinations in blank samples (without I) and found no fluorescence at the wavelength used with this method. Therefore, the method is also specific for I. Under the assay conditions which we have described, the precision and the sensitivity of the present method was found to be reliable for I determinations in some

biological liquids. It is possible to perform 14 assays in ~3 hr including reagent blanks, standards, and controls. Thus, our method should be suitable both for research in pharmacology and in a chemistry laboratory.

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Determination of Azobenzene and Hydrazobenzene in Phenylbutazone and Sulfinpyrazone Products by High-Performance Liquid Chromatography

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Received April 28, 1982, from the *Bureau of Drug Research, Health Protection Branch, Health and Welfare Canada, Tunney's Pasture, Ottawa, Ontario K1A OL2.* Accepted for publication August 27, 1982. † Deceased.

Abstract □ A high-performance liquid chromatographic method has been developed for the simultaneous determination of azobenzene and hydrazobenzene in phenylbutazone and sulfinpyrazone raw materials and formulations. The drug raw material or formulation is shaken with 1 *N* NaOH and *n*-hexane and centrifuged. The *n*-hexane layer is injected into a chromatograph equipped with a 10-μm cyano-amino bonded phase column. Azobenzene and hydrazobenzene are detected at 313 and 254 nm, respectively; the sensitivities are ~1 and 2 ppm, respectively, in the raw materials and formulations.

Keyphrases □ Azobenzene—determination in phenylbutazone and sulfinpyrazone products by high-performance liquid chromagraphy □ Hydrazobenzene—determination in phenylbutazone and sulfinpyrazone products by high-performance liquid chromatography □ High-performance liquid chromatography—determination of azobenzene and hydrazobenzene in phenylbutazone and sulfinpyrazone products

Hydrazobenzene is an intermediate in the manufacture of phenylbutazone and sulfinpyrazone (1). These drugs may be contaminated with hydrazobenzene as a result of incomplete clean up after manufacture or if the drugs degrade by hydrolytic ring opening and subsequent cleavage of the residual amido function (2). Azobenzene forms readily by autoxidation of hydrazobenzene (2); its presence in drugs may be due to the use of impure hydrazobenzene during manufacture or to the oxidation of hydrazobenzene (2). Recent work indicates that hydrazobenzene is a carcinogen in rats and mice (3) and that azobenzene is a carcinogen in rats, but not in mice (4).

There appear to be few available methods for the determination of azo- and hydrazobenzene. Hydrazobenzene (5) and azobenzene (6, 7) have been detected in phenylbutazone formulations by TLC, and a high-performance liquid chromatography (HPLC) method for the determination of hydrazobenzene in aqueous media (8) has been reported. An HPLC method for the simultaneous determination of azo- and hydrazobenzene in phenylbutazone and sulfinpyrazone drug raw materials and formulations is described here.

EXPERIMENTAL

Materials—Azobenzene[1], hydrazobenzene[2] (1,2-diphenylhydrazine), sodium hydroxide[3], HPLC-grade *n*-hexane[4], and absolute ethanol[5] were used as received. All solvents were flushed with nitrogen prior to use.

Apparatus—The liquid chromatograph consisted of a single-piston metering pump[6], an injector[7] equipped with a 100-μl sample loop, a dual-channel UV detector[8] (254 nm, 0.02 AUFS and 313 nm, 0.05 AUFS) and two 10-mV strip chart recorders[9] (chart speed of 0.5 cm/min). A Partisil-10 PAC[10] analytical column (25 cm × 4.6-mm i.d.) and a Porasil 400[11] (37–75 μm) precolumn (10 cm × 4 mm-i.d.) were used at ambient temperature with a mobile phase flow rate of 2 ml/min. The mobile phase was a solution of 2.5% absolute ethanol in *n*-hexane (v/v) flushed with nitrogen.

Calibration Curves—*Azobenzene*—A solution of azobenzene in

[1] Pfaltz and Bauer Inc., Stamford, Conn.
[2] Aldrich Chemical Co., Milwaukee, Wis.
[3] Analytical Reagent Grade.
[4] Burdick and Jackson Laboratories, Muskegon, Mich.
[5] Consolidated Alcohols Ltd., Toronto, Ont.
[6] Model 110A; Altex Scientific, Berkeley, Calif.
[7] Model CV-6-UHPa-N_{60}; Valco Instruments Co., Houston, Tex.
[8] Model 440; Waters Associates, Milford, Mass.
[9] Linear Instruments Corp., Irvine, Calif.
[10] Whatman Inc., Clifton, N.J. (Cyano-amino polar phase bonded to silica gel).
[11] Waters Associates, Milford, Mass.

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