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THE ANALYSIS OF ARILDONE IN PLASMA, URINE AND FECES BY GAS—LIQUID CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

The analysis of arildone in plasma, urine and feces by gas—liquid chromatography with electron-capture detection is described. O-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine is the derivatizing agent for the plasma and urine analysis; 3-nitrophenylhydrazine is utilized for fecal analysis. The mean (± S.E.) minimum quantifiable level of arildone was 1.4 (± 0.2) ng/ml in urine, 6.4 (± 0.1) ng/ml in plasma, and 12.6 (± 1.0) ng/g in feces. The chromatographic response was linear in the range of 0 and 10—120 ng/ml for plasma, 0 and 2.5—20 ng/ml for urine and 0 and 25—250 ng/g for feces. The estimated overall precision of the assay was 5.5%, 6.4% and 8.9% in urine, plasma and feces, respectively.

INTRODUCTION

Arildone, 4-[6-(2-chloro-4-methoxyphenoxy)hexyl]-3,5-heptanedione, is a member of a new class of antiviral agents which has shown activity against both DNA and RNA viruses; it was particularly effective against herpes simplex virus types 1 and 2 [1, 2]. Arildone has been reported to inhibit the uncoating of polio-virus in infected HeLa cells and, therefore, preventing the viral-induced inhibition of host cell protein synthesis; arildone does not inhibit either the absorption or penetration of polio-virus into the cells in tissue culture [3]. The drug is currently the subject of clinical trials to evaluate its safety and efficacy in humans.

This report describes methods for the quantitative analysis of arildone in human plasma, urine, and feces. The plasma and urine methods involve derivatization with O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine [4] and analysis

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by gas—liquid chromatography (GLC) with electron-capture (EC) detection. The fecal analysis employs 3-nitrophenylhydrazine as the derivatizing reagent prior to GLC analysis with EC detection.

EXPERIMENTAL

Materials

Arildone (Fig. 1, I) and the internal standard (Fig. 1, II) were synthesized at Sterling-Winthrop Research Institute. Hexane (ChromAR; Mallinckrodt, St. Louis, MO, U.S.A.) was distilled at atmospheric pressure before use. The O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) was synthesized at Sterling-Winthrop Research Institute or, more recently, purchased from Sigma (St. Louis, MO, U.S.A.). 3-Nitrophenylhydrazine hydrochloride (3-NPH), 98% (Aldrich, Milwaukee, WI, U.S.A.) was used as received. All other chemicals were reagent grade, with the exception of cyclohexane which was practical grade, and used without further purification.

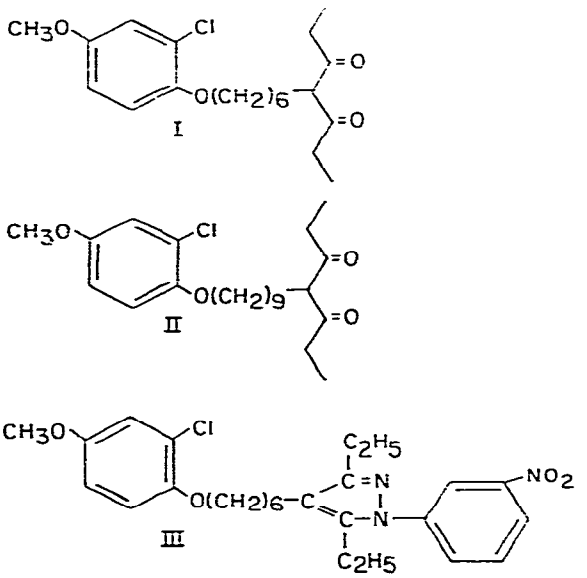

Fig. 1. Structural formulae of (I) arildone, 4-[6-(2-chloro-4-methoxyphenoxy)hexyl]-3,5-heptanedione; (II) internal standard, 4-[9-(2-chloro-4-methoxyphenoxy)nonyl]-3,5-heptanedione; (III) $C_{26}H_{32}N_3O_4Cl$ (M.W. 485).

Preparation of samples and standards

Spiked samples, to be analyzed under single-blind conditions, were prepared in human control plasma, urine and fecal homogenates [feces—triple distilled water (1:4, w/v)], coded and randomized. One set of samples in each biological medium was analyzed upon preparation; the other set was frozen for a minimum of four days at −4°C. Fresh standards, in the sample medium to be analyzed, were prepared on the day of analysis of each set of samples.

Plasma. Duplicate standards were prepared by adding appropriate volumes of an arildone stock solution (2 ng/μl in methanol) and 1.0 ml of human control

plasma (oxalate anticoagulant) to clean, unsilanized tubes to give final concentrations of 0 and 10—120 ng/ml of plasma. Two sets of quadruplicate samples were prepared, in the same manner as the standards, to give final arildone concentrations of 0, 15, 30, 52 and 84 ng/ml.

A third set of standards and samples was prepared, coded, extracted and derivatized by one analyst. The derivatized standards and samples were analyzed by GLC—EC under single-blind conditions by a second analyst. The arildone concentrations of the samples were 0, 13, 26, 32, 46 and 74 ng/ml of plasma.

Urine. Duplicate standards were prepared by adding appropriate volumes of an arildone working solution (1 ng/μl in methanol) and 2.0 ml of human control urine to clean, unsilanized tubes to give final arildone concentrations of 0 and 2.5—20 ng/ml of urine. The tubes were capped and thoroughly mixed. Two sets of triplicate samples were prepared, in the same manner as the standards, to give final arildone concentrations of 0, 3.75, 6.5, 11.0, 13.5 and 19.0 ng/ml of urine.

Feces. Duplicate standards were prepared by adding 5 ml of human control fecal homogenate (1 g feces, 4 ml triple distilled water) and appropriate volumes of an arildone stock solution (2 ng/μl in acetonitrile) to give final arildone concentrations of 0 and 25—250 ng/g of feces. The tubes were capped and thoroughly mixed. Two sets of quadruplicate samples were prepared in the same manner as the standards to give final arildone concentrations of 0, 54, 82, 106 and 178 ng/g of feces.

Assay procedure

Plasma and urine. To 1.0 ml of plasma (or 2.0 ml of urine) were added 145 ng (or 109 ng for urine) of an internal standard stock solution (145 ng per 15 μl or 109 ng per 20 μl in methanol) and 10.0 ml of hexane. The tube was shaken, centrifuged and placed in a dry ice—acetone bath to freeze the aqueous layer. The hexane was decanted into a clean tube and evaporated to dryness in a heating block with the aid of a stream of dry air. The residue was treated with 200 μl of the derivatizing reagent solution [2 mg PFBHA per 200 μl in glacial acetic acid—ethanol (5:95)]. The tube was capped, and the mixture was allowed to react for 90 min at 90°C. The reaction mixture was then evaporated to dryness in a heating block with the aid of a stream of dry air. The residue was partitioned between 200 μl of cyclohexane and 200 μl of 10% acetic acid. A 2-μl aliquot of the cyclohexane phase was analyzed on a gas—liquid chromatograph equipped with an electron-capture detector (Hewlett-Packard Model 5710A).

Feces. Sixty μl of an internal standard stock solution (435 ng per 60 μl in methanol) were added to each tube containing fecal homogenate. The contents were thoroughly mixed, and 100 μl of 5 *F* sodium hydroxide were added. The contents were thoroughly mixed, 10 ml of hexane were added, and the tube was placed on a rotary mixer for 30 min. After centrifugation, the aqueous phase was frozen in a dry ice—acetone bath, and the hexane phase was decanted into a clean tube. The hexane was evaporated to dryness at about 60°C with the aid of a stream of dry air. The residue was dissolved in 4 ml of acetonitrile and extracted three times with 2 ml of hexane. The hexane was aspirated and discarded.

To the acetonitrile phase were added 0.5 ml of 0.5 *F* ammonium hydroxide in methanol and 0.2 ml of the derivatizing solution (400 μg 3-NPH per 0.2 ml methanol). The tube was capped and heated for 15 min at 90°C. The solvent was evaporated to dryness at 60°C with the aid of a stream of dry air. The residue was partitioned between 200 μl hexane and 300 μl water. A 2-μl aliquot of the hexane phase was analyzed on a gas—liquid chromatograph, as above.

Chromatographic conditions

The column was a 2-ft. silanized glass column packed with 3% OV-1 on 100—120 mesh Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.). The column temperature was 275°C (285°C for fecal analysis), the injector and detector temperatures were 300°C, and the carrier gas was 7% methane in argon flowing at 60 ml/min. These conditions gave retention times of approximately 50 sec for derivatized I and 100 sec for derivatized II.

Extraction efficiency

The recoveries of I and II from plasma, urine and feces were determined at two or three concentrations of I by comparing the peak heights of extracted standards with those of reference standards. Reference standards were prepared by adding appropriate amounts of I and II to hexane extracts of the biological media. Extracted standards were prepared by adding appropriate amounts of I and II to control human plasma, urine and fecal homogenate and extracting them according to the procedures outlined above. The reference standards (in extracts) and extracted standards were then carried concurrently through the appropriate derivatization procedures and analyzed by GLC—EC. The per cent recovery of I was determined by comparing the peak height of the arildone derivative peak in each extracted standard with the linear regression obtained from the peak heights of the arildone derivative in the reference standards. The per cent recovery of II was calculated according to Goldstein [5] by comparing the peak heights of the internal standard derivative in the extracted samples with the peak heights of the internal standard derivative in the reference standards.

Statistical analysis

Several statistical tests were applied to the analytical data. A regression analysis of the peak height ratios (I:II) obtained for the standards was performed to determine the linearity of the response with respect to concentrations. The resulting linear regression was used to estimate the concentrations of arildone in the prepared samples. The minimum quantifiable level (*MQL*) of the assay was determined from the regression line as that concentration whose lower 80% confidence limit just encompassed zero as determined by the inverse prediction [6].

The assayed levels from the determination of the prepared samples were expressed as per cent differences from the nominal values and analyzed by a two-way analysis of variance with replication to test for a concentration effect, a time effect and a concentration—time interaction. The resulting *F*-ratios were examined for significant sources of variation. The precision of the assay was determined from this analysis.

RESULTS

Plasma and urine assay

Representative chromatograms of extracted and derivatized plasma and urine samples are shown in Fig. 2B and C. Regression analysis on the standards indicated a linear relationship between peak height ratio (derivatized I:derivatized II) over the range of 0 and 10—120 ng/ml for plasma and 0 and 2.5—20 ng/ml for urine. A summary of the results of the regression analysis is presented in Table I.

The concentrations of the prepared plasma and urine samples were estimated by inverse prediction from the appropriate regression equation and are summarized in Tables II and III, respectively. The two-way analysis of variance of the nominal values for the urine samples indicated no significant sources (concentration, time or concentration × time) of variation at $P \leqslant 0.01$. An overall estimate of the urine assay precision, based on the variance of the repeat determinations within each concentration level, was 5.5%. The accuracy of the assay, defined by the ranges of the mean per cent differences from the nominal concentration levels, varied from −13.2% to +2.1%. The mean (± S.E.) *MQL* was 1.4 (± 0.2) ng/ml, $N = 3$.

The analysis of variance on the results for the plasma samples indicated no

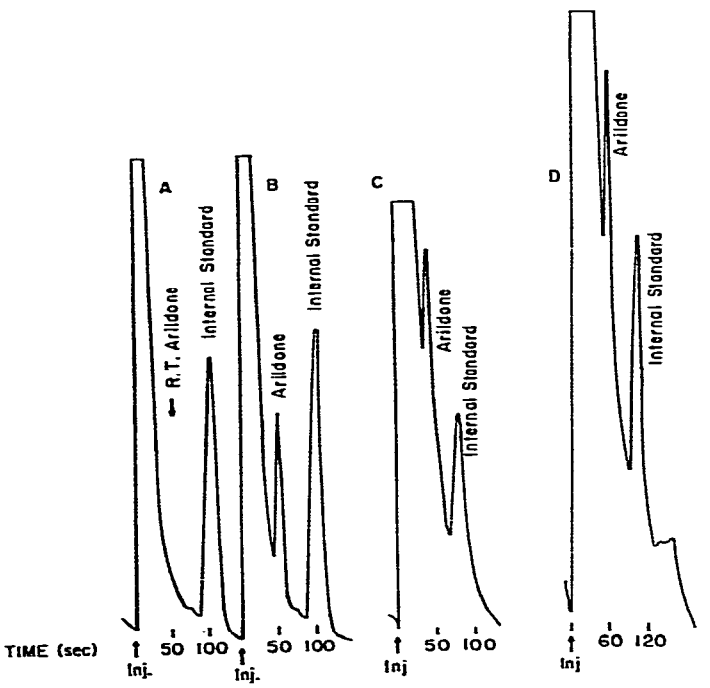

Fig. 2. Representative chromatograms (see text for chromatographic conditions). (A) Derivatized control urine extract, attenuation 128; (B) derivatized urine extract containing arildone, 11 ng/ml, attenuation 128; (C) derivatized plasma extract containing arildone, 26 ng/ml, attenuation 64; (D) derivatized fecal extract containing arildone, 100 ng/g, attenuation 128.

TABLE I

SUMMARY OF LINEAR STANDARD CURVE DETERMINATIONS

	Range (ng/ml)	No. of points	Slope* ± S.E.	Y_o** ± S.E.	*MQL* (ng/ml)
Urine					
Day 1	0—40	12	0.0318 ± 0.0010	−0.036 ± 0.023	1.2
Day 5	0—60	14	0.0284 ± 0.0009	−0.011 ± 0.028	1.7
Plasma					
Day 1	0—120	16	0.0371 ± 0.0008	−0.008 ± 0.058	5.4
Day 6	0—120	15	0.0405 ± 0.0013	0.038 ± 0.079	6.6
Day 7	0—120	16	0.0303 ± 0.0008	0.014 ± 0.051	5.9
Feces					
Day 1	0—250***	16	0.0086 ± 0.0002	−0.007 ± 0.033	13.6
Day 6	0—250	16	0.0100 ± 0.0002	−0.042 ± 0.033	11.6

*Change in peak height ratio per unit change in concentration.
**Y-axis intercept of the least-squares regression line; units are peak height ratio.
***ng/g of feces.

time effect or concentration × time interaction at $P \leqslant 0.05$; however, a significant concentration effect and a lack of agreement between the assayed and nominal values was observed. Since these observations had not been made during the development of the procedure, the third set of samples was prepared to determine if the preparation of the original samples was a significant factor. The data for this set of samples (Table II) showed no concentration effect at $P \leqslant 0.05$ and showed excellent agreement between the assayed and nominal values. The overall estimated precision of the plasma assay was 6.4% and the accuracy, based on the mean per cent differences of the assayed values from the nominal values for the last set of plasma samples, ranged from −5% to +8.5%. The mean (± S.E.) *MQL* of the plasma analysis was 6.0 (± 0.1) μg/ml, $N = 3$.

The extraction efficiency of arildone and of the internal standard was independent of the arildone concentration. From plasma, the mean extraction efficiency (± 95% confidence limits), determined at 100 ng/ml I and 290 ng/ml II, was 68 (± 24)%, $N = 4$ and 33 (± 12)%, $N = 4$ for I and II, respectively. From urine, the extraction efficiency, evaluated over the range of 5—30 ng/ml was 94.8 (± 2.0)%, $N = 14$ for I. The mean extraction efficiency for II, at a concentration of 54 ng/ml, was 86.7 (± 7.2)%, $N = 14$.

Fecal assay

A representative chromatogram of an extracted and derivatized fecal sample is shown in Fig. 2D. The regression analysis on the chromatographic peak height data for the standards indicated a linear response (Table I) in the range of 0 and 25—250 ng/g feces. The concentrations of the prepared samples, estimated from the regression analysis, are summarized in Table IV. No significant sources of variation were observed at $P \leqslant 0.05$. The estimated overall assay precision was 8.9% and the accuracy of the assay ranged from −9.2% to +1.6%. The mean (± S.E.) *MQL* of the fecal analysis was 12.6 (± 1.0) ng/g.

TABLE II

SUMMARY OF DATA FROM ANALYSIS OF ARILDONE IN PREPARED PLASMA SAMPLES (ng/ml)

Concentration level	Assayed level*	Assayed level**	Concentration level§§§	Assayed level§§§
0	<*MQL****	<*MQL*§	0	<*MQL*†
	<*MQL*	<*MQL*		<*MQL*
	<*MQL*	<*MQL*		<*MQL*
	<*MQL*	<*MQL*		
15	13.7	9.6	13	14.0
	11.5	11.6		12.7
	11.8	12.1		14.0
	11.5	13.8		
Mean	12.1	11.8	Mean	13.6
S.E.M. (%)	4.4	7.3	S.E.M. (%)	3.2
Mean difference (%) from freshly prepared samples		−2.5	Mean difference (%) from nominal	+4.4
30	25.5	29.3	26	26.9
	26.6	—§§		29.2
	27.7	27.8		28.5
	26.6	28.8		
Mean	26.6	28.6	Mean	28.2
S.E.M. (%)	1.7	1.3	S.E.M. (%)	2.4
Mean difference (%) from freshly prepared samples		+7.5	Mean difference (%) from nominal	+8.5
52	44.4	49.0	32	37.1
	49.2	46.5		29.9
	48.7	42.4		30.5
	45.5	48.8		
Mean	47.0	46.7	Mean	32.5
S.E.M. (%)	2.5	3.3	S.E.M. (%)	7.1
Mean difference (%) from freshly prepared samples		−0.6	Mean difference (%) from nominal	+1.6

(Continued on p. 220)

TABLE II (continued)

Concentration level	Assayed level*	Assayed level**	Concentration level§§§	Assayed level§§§
84	92.9	77.8	46	42.7
	88.3	86.6		—§§
	96.9	92.6		44.7
	92.1	85.4		
Mean	92.6	85.6	Mean	43.7
S.E.M. (%)	1.9	3.6	S.E.M. (%)	2.3
Mean difference (%) from freshly prepared samples		—7.6	Mean difference (%) from nominal	—5.0
			74	76.0
				77.6
				79.3
				77.3
			Mean	77.6
			S.E.M. (%)	0.8
			Mean difference (%) from nominal	+4.8

*Assayed immediately after preparation.
**Frozen for 5 days before analysis.
***MQL = 5.4 ng/ml.
§MQL = 6.6 ng/ml.
§§ Sample lost.
§§§Samples and standards prepared by same analyst.
†MQL = 5.9 ng/ml.

TABLE III

SUMMARY OF DATA FROM ANALYSIS OF ARILDONE IN PREPARED URINE SAMPLES (ng/ml)

Concentration level	Assayed level*	Assayed level**
0	<*MQL****	<*MQL*§
	<*MQL*	<*MQL*
	<*MQL*	<*MQL*
	<*MQL*	<*MQL*
3.75	3.5	3.2
	3.7	3.4
	3.4	3.2
Mean	3.5	3.3
S.E.M. (%)	2.5	2.0
Mean difference (%)	−5.6	−13.2
6.5	5.4	5.8
	6.5	5.7
	5.1	6.0
Mean	5.7	5.8
S.E.M. (%)	7.5	1.5
Mean difference (%)	−12.6	−10.2
11.0	10.8	9.4
	10.0	9.7
	10.6	10.2
Mean	10.5	9.8
S.E.M. (%)	2.3	2.4
Mean difference (%)	−5.0	−11.1
13.5	12.6	15.0
	13.3	13.4
	13.1	12.9
Mean	13.0	13.8
S.E.M. (%)	1.6	4.6
Mean difference (%)	−3.6	+2.1
19.0	19.1	18.7
	17.5	18.2
	17.8	19.8
Mean	18.1	18.9
S.E.M. (%)	2.7	2.5
Mean difference (%)	−4.5	−0.5

*Assayed immediately after preparation.
**Frozen for 4 days before analysis.
***MQL = 1.2 ng/ml.
§MQL = 1.7 ng/ml.

TABLE IV

SUMMARY OF DATA FROM ANALYSIS OF ARILDONE IN PREPARED HUMAN FECAL SAMPLES (ng/g OF FECES)

Concentration level (ng/g)	Assayed level fresh*	Assayed level frozen**
0	<*MQL****	<*MQL*§
	<*MQL*	<*MQL*
	<*MQL*	<*MQL*
	<*MQL*	<*MQL*
54	54	50
	58	37
	47	53
	55	56
Mean	54	49
S.E.M. (%)	4.4	8.5
Mean difference (%)	−0.9	−9.2
82	84	82
	82	82
	72	76
	89	77
Mean	82	79
S.E.M. (%)	4.4	2.0
Mean difference (%)	−0.3	−3.4
106	98	96
	96	106
	115	114
	110	103
Mean	105	105
S.E.M. (%)	4.4	3.6
Mean difference (%)	−1.2	−1.2
178	172	180
	178	175
	168	166
	205	176
Mean	181	174
S.E.M. (%)	4.6	1.7
Mean difference (%)	+1.5	−2.1

*Analyzed upon preparation.
**Frozen for 5 days before analysis.
***MQL = 13.6 ng/g.
§MQL = 11.6 ng/g.

The mean (± S.E.) extraction efficiency for I over the range of 25—250 ng/g was 100 (± 2)%, N = 15. The mean (± 95% confidence limit) recovery for II (290 ng/g) was 77.8 (± 10.4)%.

DISCUSSION

Arildone presented several challenges for analytical method development.

The anticipated levels in biological media are in the ng/ml (or ng/g) range or lower, necessitating a highly sensitive method. This was complicated by the neutral character of the molecule which precluded the use of a back-extraction for elimination of interferences. In addition, derivatization of the β-diketone groups generally led to the formation of a mixture of *cis*- and *trans*-isomers. In the plasma and urine procedures, the PFBHA derivatization gave rise to two chromatographic peaks for both I and II. The use of the short GLC column merged the peaks due to isomers so that single peaks were observed for each I and II.

Binding to glassware and the GLC column was observed for both arildone and the internal standard. Use of silanized glassware aggravated the problem, but the presence of plasma or a plasma extract minimized binding to glassware. The GLC column required conditioning by injecting several samples containing high concentrations of the derivatives prior to injection of the standards and samples.

The assay has been useful for the analysis of arildone in human, rabbit, monkey or rat plasma; human or rat urine; and human feces. The analysis of dog plasma, however, revealed an interfering peak with the retention time of the derivative of the internal standard, II. For the analysis of dog plasma, 4-[6-(2-chloro-4-methoxyphenylamino)hexyl]-3,5-heptanedione, may be used as the internal standard.

In the analysis of fecal samples, PFBHA gave numerous derivatives of endogenous fecal components which could not be removed from the sample and which interfered with the GLC analysis. Derivatization of I with 3-NPH gave a single derivative identified by mass spectrometry as having a molecular weight corresponding to Fig. 1, III. This derivatization, coupled with the hexane—acetonitrile step in the clean-up allowed the analysis of fecal samples at levels as low as 15 ng/g of feces.

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