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Isatin (indole-2,3-dione) in urine and tissues

Detection and determination by gas chromatography–mass spectrometry

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ABSTRACT

A simple procedure based upon capillary column gas chromatography-mass spectrometry (GC–MS) is described for the detection and determination of isatin (indole-2,3-dione) in body fluids and tissues. After addition of 5-methylisatin as internal standard to urine or tissue homogenates, organic extracts are dried and derivatized successively with hydroxylamine hydrochloride and the reagent N-*tert.*-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA). The *tert.*-butyldimethylsilyl derivatives obtained show good GC–MS properties and allow quantification by selected-ion monitoring of *m/z* 333 (isatin) and *m/z* 347 (internal standard). Adult and newborn human urine output values lie in the ranges 0.4–3.2 mg/mmol of creatinine (5–30 mg per 24 h) and 0.002–0.518 mg/mmol of creatinine, respectively. There is a discontinuous regional distribution in rat tissues. The GC–MS properties of a number of derivatives formed by successive reaction of isatin with hydroxylamine hydrochloride (or methoxyaminehydrochloride or ethoxyamine hydrochloride) and MTBSTFA, bis(trimethylsilyl)trifluoroacetamide, pentafluoropropionic anhydride or pentafluorobenzyl bromide are also described.

INTRODUCTION

Although isatin was first synthesized in 1841 [1] and early characterized as a derivative of indigo (Fig. 1), its presence in mammalian urine and tissues has only recently been demonstrated [2]. This latter investigation also showed that isatin accounts for a proportion of the activity of tribulin, an endogenous monoamine

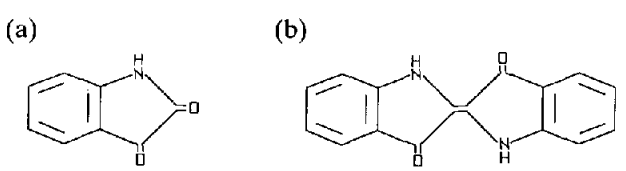

Fig. 1. Structures of (a) isatin and (b) indigo.

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oxidase inhibitor which had previously been purified [3]. Isatin is a well known pharmacological agent with properties which appear to fall into two main categories, a range of actions in the central nervous system and protection against certain types of infection or infestation. Its pharmacological effects include inhibition of monoamine oxidase – its most powerful *in vitro* action found to date [2] – and of alkaline phosphatase [4]. It increases vigilance [5], reduces slow-wave sleep [6], potentiates the anti-seizure action of propranolol [7], selectively damages *Echinococcus multilocularis* [8], possesses antiviral activity [9] and is generated by certain commensal bacteria to protect shrimp embryos against a pathogenic fungus [10]. Recent work has demonstrated a discontinuous tissue distribution in the rat [11]. Its known properties have recently been reviewed [12].

The present paper described for the first time the gas chromatographic–mass spectrometric (GC–MS) properties of a number of isatin derivatives. A simple procedure combining oxime formation and reaction with N-*tert.*-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) [13] is described for the determination of isatin in urine and tissues by selected-ion monitoring (SIM). The ready availability of low-cost bench-top GC–MS systems no longer precludes the application of GC–MS on cost grounds. This specific and sensitive method allows the measurements to be carried out with minimal sample preparation and should assist further in the exploration of this new biological factor.

EXPERIMENTAL

Materials

Isatin was obtained from Aldrich (Poole, U.K.) and recrystallized before use. 5-Methylisatin was synthesized by the method of Sandmeyer [14]. Isatin-2-oxime was prepared from diacetylindoxyl by treatment with nitrous acid followed by acid hydrolysis [15]. Silylation reagents employed were: bis(trimethylsilyl)-trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) (Sigma, Poole, U.K.) and MTBSTFA containing 1% *tert.*-butyldimethylchlorosilane (TBDMCS) (Fluka, Glossop, U.K.). Pentafluoropropionyl anhydride (PFPA) and pentafluorobenzyl (PFB) bromide were purchased from Sigma and Fluka, respectively. Solvents were of analytical grade (BDH, Poole, U.K.) and silicic acid (Bio-Sil A, 100–200 mesh) was obtained from Bio-Rad, (Richmond, CA, U.S.A.).

Urine extraction and oxime formation

Urine (0.25 ml) was treated with solid sodium bicarbonate to raise the pH above 7, followed by 50 μl of hydroxylamine · HCl or methoxyamine · HCl or ethoxyamine · HCl solution (20 mg/ml in water) and 100 μl of 5-methylisatin solution (10 μl/ml in ethanol). The mixture was left overnight at room temperature, two drops of concentrated HCl were added and the whole was transferred to a short column of silicic acid (4 cm height in a Pasteur pipette loosely plugged

with glass wool). The isatin oxime was extracted by washing the column with 1.5 ml of ethyl acetate.

Tissue extraction and oxime formation

Typically, 1 g wet weight of tissue was homogenized (4°C) with 3 ml of 1 *M* HCl and 10 μl of 5-methylisatin solution (10 μg/ml in ethanol). After centrifugation (4°C) at 12 500 *g* for 15 min, the supernatant was washed with 4 ml of *n*-heptane extracted with 2.5 ml of ethyl acetate and dried down under nitrogen. Hydroxylamine · HCl solution (50 μl, or methoxyamine · HCl or ethoxyamine · HCl, 20 mg/ml in water) was added and the mixture left overnight at room temperature.

Silylation

After drying down under a stream of dry nitrogen, the oxime residue was treated with 50 μl of acetonitrile and 50 μl of silylating reagent (BSTFA–TMCS or MTBSTFA–TBDMCS) in the urine case and half these volumes in the case of tissue extracts. The silylation reactions were allowed to proceed for at least 1 h at room temperature before GC–MS analysis. A 1-μl volume of reaction mixture was injected in the splitless mode.

Oxime-pentafluoropropionyl

Oxime formation was carried out as described above, except that 50 μg of isatin were used. The dry residue was heated at 70°C with a mixture of 100 μl of PFPA and 20 μl of acetonitrile for 1 h. After evaporation to dryness, the derivative was reconstituted in 50 μl of *n*-heptane before analysis by GC–MS.

Oxime-pentafluorobenzyl

The dry oxime residue from 50 μg of isatin was treated with 50 μl of a mixed base solution (5% KOH and 5% triethylamine in ethanol) and 50 μl of a 20% PFB solution. After heating for 1 h at 70°C, the solvents were removed under a stream of dry nitrogen and 50 μl of ethyl acetate added to dissolve the derivative prior to GC–MS analysis.

Gas chromatography–mass spectrometry

GC–MS was performed with a Hewlett-Packard 5970B MSD bench-top quadrupole connected to a Hewlett-Packard 5711A gas chromatograph via a direct capillary interface maintained at 250°C. The column used was a Hewlett-Packard 25 m × 0.22 mm I.D. fused silica with chemically bonded HP-1 stationary phase (equivalent to OV-1) and film thickness 0.33 μm. For spectrum scanning and calculation of retention data (see below), the oven was programmed from 100 to 280°C at 10°C/min but a faster gradient was employed during SIM: 140 to 280°C at 30°C/min. Splitless injection mode was used thoughout and the scanned mass range was *m/z* 33–450 or 33–550.

In the SIM mode, four ions were monitored: *m/z* 333, 334, 347 and 348. The dwell-time on each ion was 50 ms. Data was acquired with a Hewlett-Packard 59970C Chemstation.

Quantification of isatin was based upon the ratio of peak areas for the TBDMS derivative of the isatin-3-oxime (*m/z* 333) to those of the internal standard, 5-methylisatin (*m/z* 347). Areas were also measured for *m/z* 334 and 348 as an additional specificity control. Retention indices in the form of methylene unit (MU) values were measured relative to a homologous series of co-injected *n*-alkanes.

Infrared (IR) spectrophotometry was performed in KBr discs using a Pye Unicam SP1000 instrument.

RESULTS AND DISCUSSION

Silicon-containing derivatives

An overview of the major GC–MS characteristics of the mixed silyl derivatives of isatin is given in Table I. The mass spectral patterns obtained are as generally expected and a small molecular ion was found in each case. The base peak in all spectra of the silylated mixed derivatives was *m/z* 73. Methoxyamine and ethoxy-

TABLE I

METHYLENE UNITS (MU, OV-1) AND 70-eV MASS SPECTRAL DATA OF MIXED ISATIN DERIVATIVES OXIME-TMS and OXIME-TBDMS

3-Oxime			Silyl		MU (OV-1)	Major ions > *m/z* 80[a]							
–OH	–OMe	–OEt	TMS	TBDMS									
–	–	–	+	–	16.8	90	149	176	219	92	191	117	204
						(16)	(15)	(15)	(9)	(8)	(7)	(6)	(4)
+	–	–	+	–	19.0	147	217	84	144	89	306	291	203
						(10)	(9)	(4)	(3)	(3)	(2)	(2)	(2)
–	+	–	+	–	18.2	217	89	174	84	144	116	162	248
						(23)	(16)	(9)	(9)	(8)	(8)	(7)	(6)
–	–	+	+	–	18.8	217	84	162	174	262	144	116	203
						(45)	(15)	(12)	(10)	(9)	(9)	(8)	(7)
–	–	–	–	+	18.3	204	176	205	90	121	146	148	261
						(46)	(23)	(21)	(12)	(5)	(4)	(3)	(1)
+	–	–	–	+	22.4	333	147	203	133	259	105	277	390
						(71)	(50)	(25)	(15)	(11)	(11)	(6)	(1)
–	+	–	–	+	19.6	233	174	89	84	203	202	116	290
						(99)	(99)	(80)	(44)	(37)	(32)	(13)	(4)
–	–	+	–	+	20.2	247	174	84	203	103	219	202	304
						(64)	(46)	(23)	(19)	(15)	(11)	(9)	(1)

[a] Molecular ions are underlined; values in parentheses are percentages base peaks.

amine mixed silyl derivatives had fairly abundant M − 31 and M − 45 ions, respectively. The corresponding M − 89 ion in the mass spectrum of the oxime-N,O-bis(trimethylsilyl) derivative was of low abundance. All mixed derivatives containing the TBDMS group had spectra dominated by M − 57 ions. The simple trimethylsilylation to give the N-trimethylsilyl (TMS) derivative (MW = 219) was found to be unsatisfactory at an early stage [2]. No serious instability was encountered with the oxime mixed derivatives.

The TBDMS derivative of the oxime was chosen for further work as having an abundant high-mass ion (*m/z* 333). The line diagram mass spectra for this deriv-

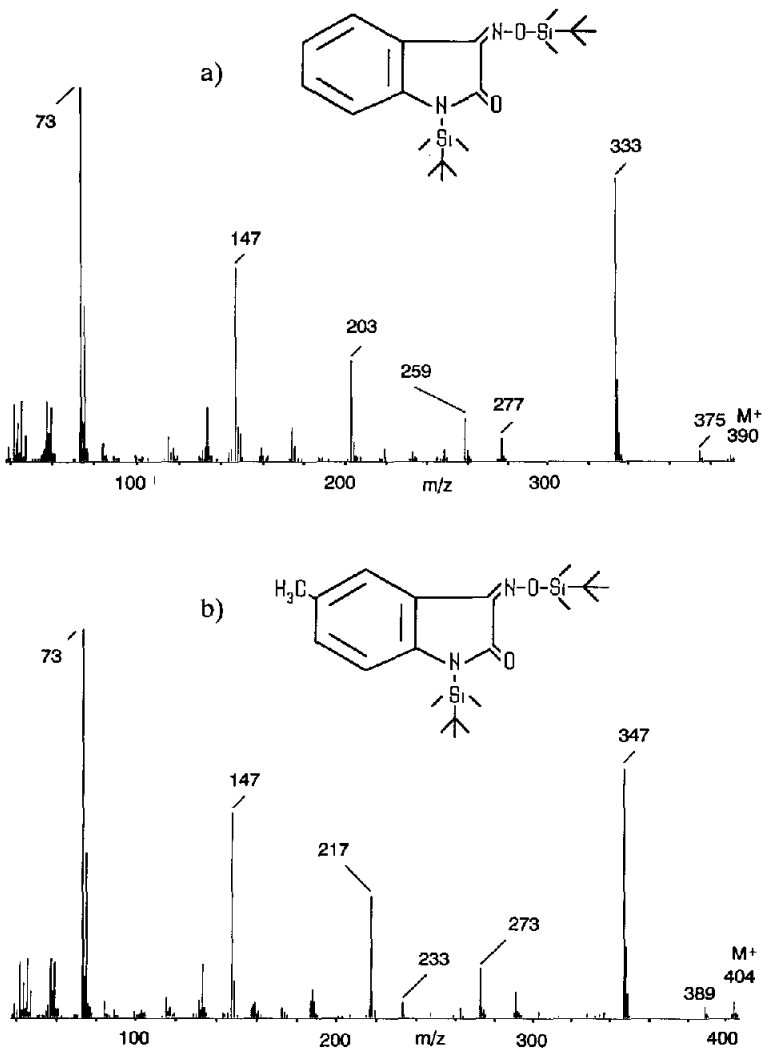

Fig. 2. Mass spectra (70 eV) obtained during GC–MS analysis of a standard mixture of (a) isatin and (b) 5-methylisatin (internal standard) N-*tert.*-butyldimethylsilyl-3-oxime-*tert.*-butyldimethylsilyl derivatives.

ative and for the internal standard, together with their probable structures, are shown in Fig. 2. The MU value for the latter was found to be 23.19, *i.e.* the isatin derivative had a relative retention time of 0.95 to the internal standard derivative. Molecular ions of low abundance are present in both cases and, as expected, the M − 57 ions were found to predominate, making these derivatives particularly suitable for SIM.

The presence of a keto group in the derivative was demonstrated by IR spectrophotometry. The spectrum of the derivative was found to have a large carbonyl band shifted to 1737 cm^{-1} relative to that of the unsilylated 3-oxime (1717 cm^{-1}) and the MTBSTFA reagent (1705 cm^{-1}).

The mass spectrum of the 3-oxime derivative also differed from that of the 2-oxime derivative, the latter having an MU value of 21.8 and *m/z* 333 as base peak. Other significant ions (percentage base peak in parentheses) were *m/z* 73 (39), 147 (18), 219 (11), 275 (8), 389 (0.8) and the molecular ion *m/z* 390 (0.6).

The total ion current chromatogram obtained by GC–MS of an extract of adult urine after oxime-TBDMS derivatization is reproduced in Fig. 3. Although a very minor peak in this chromatogram, the presence of isatin is clearly indicated by the peak at a retention time of 12.25 min in the reconstructed ion chromatogram for *m/z* 333. A full spectrum identical to that of the standard isatin derivative was obtained at this position.

Fluorine-containing derivatives

An overview of the major GC–MS characteristics of the mixed pentafluoropropionyl (PFP) and PFB derivatives of isatin is given in Table II. No satis-

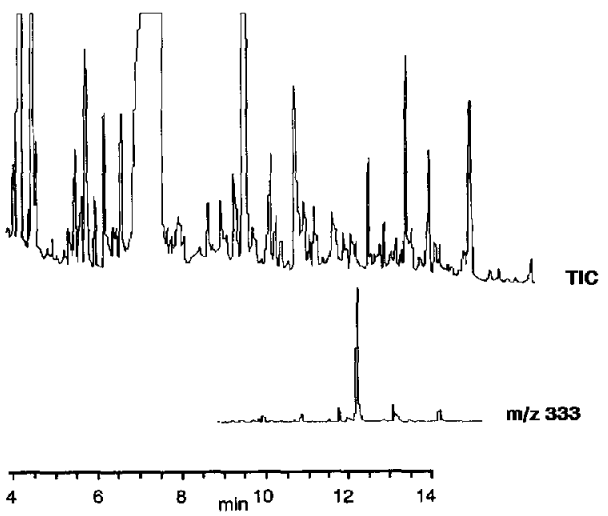

Fig. 3. Profile of adult urine extract prepared as the 3-oxime-*tert.*-butyldimethylsilyl derivative. Top, total ion current (TIC) chromatogram; bottom, SIM chromatogram (*m/z* 333) demonstrating the presence of the isatin derivative (90 ng) at a retention time of 12.25 min (MU = 22.38).

TABLE II

METHYLENE UNITS (MU, OV-1) AND 70-eV MASS SPECTRAL DATA OF MIXED ISATIN DERIVATIVES OXIME-PFP and OXIME-PFB

3-Oxime			PFP	PFP	MU (OV-1)	Major ions > m/z 80[a]							
–OH	–OMe	–OEt											
–	–	–	+	–	14.6	90	146	119	265	293	218	246	198
						(100)	(91)	(13)	(6)	(1)	(<1)	(<1)	(<1)
–	+	–	+	–	16.7	119	144	116	160	322	195	263	264
						(100)	(95)	(59)	(31)	(22)	(12)	(6)	(5)
–	–	+	+	–	17.4	119	144	116	250	145	133	336	308
						(100)	(63)	(48)	(24)	(34)	(24)	(18)	(9)
–	–	–	–	+	20.7	90	252	146	181	327	161	270	299
						(100)	(60)	(45)	(40)	(21)	(19)	(11)	(5)
+	–	–	–	+	27.0	181	161	522	505	–	–	–	–
						(100)	(7)	(1)	(1)	–	–	–	–
–	+	–	–	+	22.2	181	103	179	102	161	297	356	144
						(100)	(17)	(16)	(14)	(13)	(11)	(10)	(10)
–	–	+	–	+	22.9	181	297	145	103	370	161	102	342
						(100)	(13)	(13)	(13)	(11)	(11)	(11)	(1)

[a] Molecular ions are underlined; values in parentheses are percentages base peak.

factory spectrum was obtained from the product of reaction of isatin with hydroxylamine · HCl and PFPA. The oxime-PFP mass spectra exhibit molecular ions having abundance approximately 20% of the base peak which is at m/z 119 and corresponds to the C_2F_5 ion. This latter fragmentation dominates the spectra, and the M − 31 and M − 45 ions are very small in the methoxime and ethoxime cases, respectively.

The oxime-PFB spectra were dominated by m/z 181 peaks which correspond to the pentafluorobenzyl ion. Molecular ions were found to be very small. They were therefore considered to be less suitable for SIM.

Quantification in urine

Calibration graphs for the TBDMS derivative of isatin-3-oxime were found to be linear within the range employed for urine: 0, 1, 2, 4 and 6 μg/ml ($r = 0.9950$). Larger concentrations in certain samples were calculated by extrapolation. Recoveries were estimated by treatment of water blanks to which known amounts of isatin were added ($n = 3$) in triplicate in the same manner as urine samples. Values (mean ± S.D.) measured for the actual values 0.40, 2.40 and 4.80 μg/ml were (percentage recovery in parentheses) 0.32 ± 0.05 (80%), 2.39 ± 0.09 (100%) and 5.07 ± 0.02 μg/ml (106%), respectively. The limit of detection of the method was approximately 2 ng/ml.

Values found in the five adult urine samples investigated ranged from 5 to 30

mg per 24 h (mean ± S.D. 14.6 ± 9.9 mg per 24 h). Values found in newborns (42 samples from 8 babies) were in the range 2–518 ng/μmol of creatinine (mean ± S.D. 159 ± 143 ng/μmol of creatinine). The possible clinical relevance of the urinary excretion values is currently under investigation.

Quantification in tissues

A typical example of the SIM chromatograms (*m/z* 333, 334, 347 and 348) obtained from a rat brain sample (TBDMS derivative of isatin-3-oxime) are shown in Fig. 4. The amount of isatin in this case corresponds to 170 pg injected.

Calibration graphs were found to be linear within the range employed: 0, 25, 50, 100 and 150 ng per sample ($r = 0.9936$). Recoveries were estimated by treatment of 3 ml of spiked water blanks ($n = 3$) in triplicate in a manner identical to that for homogenized tissue samples. Values (mean ± S.D.) measured for the theoretical values 20, 60 and 120 ng were (percentage recovery in parentheses) 16 ± 2 (80%), 57 ± 1 (95%) and 127 ± 6 ng (106%), respectively.

Results of determinations (mean ± S.D.) on a number of rat tissues were: brain, 38 ± 7 ng/g ($n = 7$); heart, 45 ± 13 ng/g ($n = 6$); liver, 29 ± 11 ng/g ($n = 6$); kidney, 69 ± 23 ng/g ($n = 6$) and vas deferens, 284 ± 190 ng/g ($n = 5$). The limit of detection was approximately 5 ng/g.

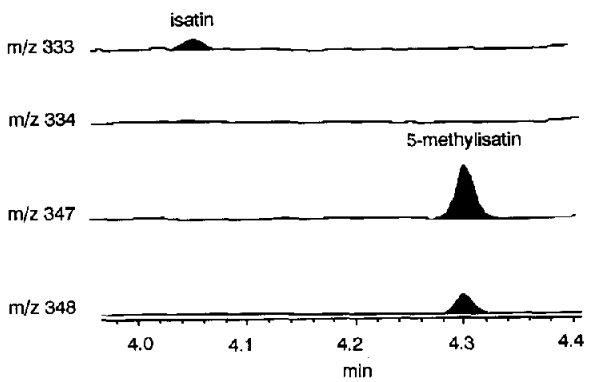

Fig. 4. SIM chromatograms obtained from a derivatized whole rat brain extract: *m/z* 333 and *m/z* 334 (isatin-oxime-TBDMS), *m/z* 347 and 348 (5-methylisatin-oxime-TBDMS, internal standard). The amount of isatin injected into the GC–MS instrument in this case corresponds to 170 pg.

CONCLUSION

The present results provide a further example of the utility of TBDMS derivatives in GC–MS both for detection and determination of a substance of interest. Useful quantitative data are rapidly obtained with minimal time-consuming sample preparation, even from small amounts of rat tissue. MS and GC–MS will

continue to play an important role in the study of the occurrence of isatin in the organism and contribute to the investigation of its origin and fate.

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