parameters in the ecology of lichens and may equally be important for other poikilohydric plants.

J. D. MACFARLANE
K. A. KERSHAW
Department of Biology,
McMaster University,
Hamilton, Ontario, Canada L8S 4K1

References and Notes

1. W. Zopf, in *Handbuch der Botanik*, A. Schenk, Ed. (Trendwendt, Breslau, 1890), vol. 4, pp. 271–755; J. F. Farrar, in *Air Pollution and Lichens*, B. W. Ferry, M. S. Baddeley, D. L. Hawksworth, Eds. (Athlone, London, 1973), pp. 266–267; W. Larcher, *Physiological Plant Ecology* (Springer Verlag, Berlin, 1975), p 212; P. H. Raven, R. F. Evert, H. Curtis, *Biology of Plants* (Worth, New York, 1976), p. 230.
2. O. L. Lange, *Flora (Jena)* **140**, 39 (1953); *ibid.* **142**, 381 (1955); *Arch. Meteorol. Ser. B* **5**, 182 (1953); *Arch. Meteorol. Geophys. Bioklimatol. Ser. B* **5**, 182 (1954).
3. J. D. MacFarlane and K. A. Kershaw, *New Phytol.* **79**, 403 (1977).
4. D. W. Larson and K. A. Kershaw, *Can. J. Bot.* **53**, 1535 (1975).
5. K. A. Kershaw, *New Phytol.* **79**, 391 (1977).
6. J. Levitt, *Responses of Plants to Environmental Stresses* (Academic Press, New York, 1972), pp. 299–321; R. Geiger, *The Climate Near the Ground* (Harvard Univ. Press, Cambridge, Mass., 1965), pp. 258–297.
7. L. Kappen, in *The Lichens* (Academic Press, New York, 1973), pp. 311–380.
8. J. D. MacFarlane and K. A. Kershaw, in preparation.

2 March 1978; revised 17 April 1978

Analysis of Melatonin in Human Plasma by Gas Chromatography Negative Chemical Ionization Mass Spectrometry

Abstract. *Three techniques have been used to measure human plasma melatonin: bioassay, radioimmunoassay, and gas chromatography–mass spectrometry (GC-MS). GC-MS is theoretically capable of the greatest specificity, but in general suffers from insufficient sensitivity. Negative chemical ionization, a new technique, provides a 150-fold increase in GC-MS sensitivity for electron-capturing compounds. Negative chemical ionization GC-MS permits routine measurement in human plasma of melatonin at a concentration as low as 1 picogram per milliliter.*

Measurement of melatonin in human plasma has become possible only within the last few years (*1–10*). Extremely low circulating concentrations (1 to 10 pg/ml or 1 to 10 parts per trillion) permit measurement by only three techniques: bioassay (*4, 5*), radioimmunoassay (RIA) (*3, 6–9*), and gas chromatography–mass spectrometry (GC-MS) (*10*). Quantification at parts per trillion contends with problems of contamination and nonspecific interference. Theoretically, GC-MS offers the greatest specificity since quantified substances are simultaneously identified (by retention time and molecular weight of fragment ions). Newer GC-MS instrumentation offers very high sensitivity for the detection of trace organic compounds; however, when used for quantification of endogenous compounds in a biological matrix, problems of increased chemical background and irreversible adsorption phenomena limit application of these methods to parts per billion (ng/g). A new technique, negative chemical ionization (CI) mass spectrometry, provides the requisite sensitivity and specificity to measure melatonin at concentrations present in human plasma.

The potential sensitivity of negative CI GC-MS has been demonstrated by Hunt (*11*) who showed a 10- to 100-fold sensitivity enhancement for electron-capturing compounds. Appropriately derivatized organic compounds undergo efficient ionization by resonance capture of a near thermal energy electron in an exothermic process. Excess energy is dissipated by fragmentation or collisional stabilization. Cyclic derivatives or polycyclic compounds generally yield structurally specific anions, whereas acyclic derivatives with good anion leaving groups (such as perfluoracyl derivatives of aliphatic alcohols) generate only reagent specific ions.

Quantification of biological compounds in picogram and femtogram amounts required special precautions. Reagents were of high purity (*12*). Glassware was acid washed and silanized with hexamethyldisilazane in a heated vacuum desiccator (*13*).

Blood was collected with the use of silanized glassware and stainless steel needles with heparin or EDTA as anticoagulant and immediately centrifuged at 5000 rev/min at 4°C. Samples of plasma could then be assayed either directly or frozen for subsequent analysis. To 1 ml of plasma was added a tetradeuterated internal standard consisting of 15 to 40 pg of *N*-acetyl-5-methoxy-[$\alpha,\alpha,\beta,\beta$,-$D_4$]tryptamine synthesized by Shaw *et al.* (*14*), which extracts, derivatizes, and chromatographs with nondeuterated melatonin. Deuterated melatonin was also added to saline standards containing 1 to 200 pg of nondeuterated melatonin. The ratio of nondeuterated melatonin (endogenous or saline standard) to deuterated melatonin (internal standard) corrected for recovery (which could vary as much as fivefold).

To 1 ml of plasma or saline standard (containing internal standard) was added an equal volume of 0.5*M* borate buffer

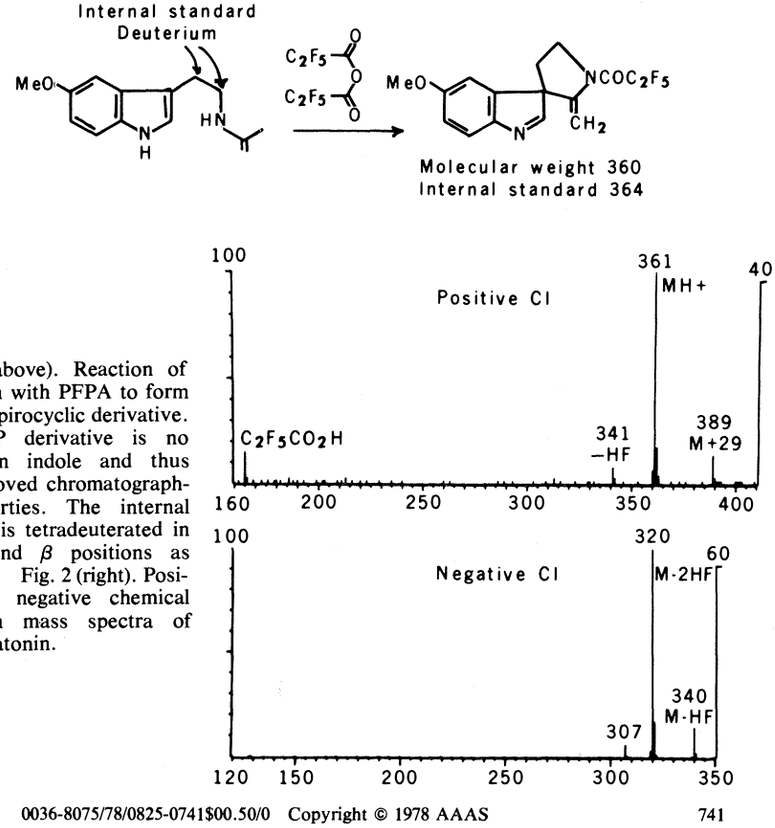

Fig. 1 (above). Reaction of melatonin with PFPA to form a stable spirocyclic derivative. The PFP derivative is no longer an indole and thus has improved chromatographic properties. The internal standard is tetradeuterated in the α and β positions as shown. Fig. 2 (right). Positive and negative chemical ionization mass spectra of PFP-melatonin.

0036-8075/78/0825-0741$00.50/0

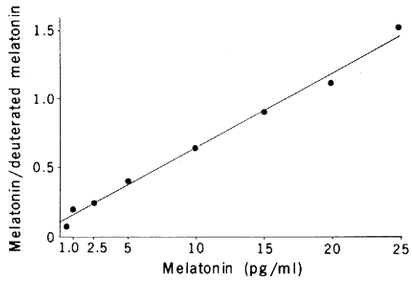

Fig. 3. Standard curve prepared from 1 ml of aqueous standards containing 19 pg/ml of tetradeuterated internal standard. The regression equation is $Y = 0.111 + 0.0538X$, with a correlation coefficient $r = .997$.

(*p*H 10), saturated with KCl; this mixture was gently shaken with ten volumes of petroleum ether to remove lipids and other substances and centrifuged; the organic phase was removed, and melatonin was extracted into ten volumes of chloroform by gentle shaking; the aqueous phase was discarded, and the organic fraction was transferred to a fresh tube, and then evaporated to dryness under nitrogen. Benzene (1 ml) was added to the residue and this solution was dried with nitrogen to remove traces of water that would interfere with derivatization.

The dried extract was dissolved in 0.4 ml of dry acetonitrile. Derivatization was performed with 25 μl of pentafluoropropionic anhydride (PFPA) catalyzed with 5 percent trimethylamine in dry benzene (0.5 ml) at 75°C for 15 minutes. The structure (Fig. 1) of this product is a singularly stable spirocyclic compound, recently described by Blau *et al.* (*15*). The incorporated fluorine atoms provide improved electron-capturing properties essential for negative CI with high sensitivity.

The reaction product (PFP-melatonin) was washed by vigorous shaking, first with 1 ml of water and then with 1 ml of 5 percent NH_4OH. After centrifugation, the organic phase was removed and evaporated to dryness under nitrogen. The dried extract was partitioned between 0.5 ml of acetonitrile and 1 ml of hexane by vigorous shaking and centrifugation. After the hexane layer was removed, the acetonitrile was blown to dryness with nitrogen. Partitioning was repeated a second time. The dried derivative was quite stable and could be kept at −20°C for more than several weeks. Washing the derivative protected the GC column from destruction by unreacted PFPA; partitioning the derivative reduced interference from electron-capturing background that might elute with the solvent and obscure the sample peaks.

Before injection onto the GC, the dried derivative was dissolved in 15 μl of ethyl acetate. Approximately half of this volume was injected into a glass column (5 m by 2 mm, inside diameter) packed with 1 percent OV-225 on 100- to 120-mesh Gas-Chrom Q (Applied Science) operated at 235°C with methane as carrier gas. The melatonin eluted after 4 to 6 minutes; the column effluent was diverted from the ion source for the first 2 to 3 minutes. Mass spectra were recorded with an Extranuclear SpectrEl electronics and a Finnigan 3200-GC-CI analyzer. A Cu-Be electron multiplier (F4074, ITT) was used as described by Stafford (*16*) with the first (conversion) dynode operating at +2 kV, the second dynode at −2.0 to −2.5 kV, and the signal referenced to ground. Figure 2 shows the positive and negative CI mass spectra of PFP-melatonin. Tetradeuterated melatonin showed the expected shift of 4 atomic mass units by positive CI analysis. However, negative CI revealed a shift of 3 atomic mass units for the fragment ions *m/e* (mass to charge) 320 and 340, consistent with the sequential losses of DF and HF from the molecular anion. The *m/e* 320 in the negative spectrum was eight times more intense than *m/e* 340 and 150 times more intense than *m/e* 361 (protonated molecular ion) in the positive spectrum. Thus, the negative spectrum permits a highly sensitive detection of melatonin at *m/e* 320, as well as measurement of *m/e* 340 for confirmation of assay specificity.

Fig. 4. Negative chemical ionization selected ion recording of an extract of 1 ml of human plasma, with an internal standard (19 pg/ml) (*m/e* 323) and endogenous melatonin (50 pg/ml) with mass fragment ions of *m/e* 340 and *m/e* 320 (attenuated, × 2.5).

Table 1. Range of concentrations of melatonin in human plasma or serum. Some values were estimated from graphs. Only Smith *et al.* (*9*) and the GC-MS techniques have corrected for recovery.

Nadir (pg/ml)	Zenith (pg/ml)	Method
	Bioassay	
0	28–84	Pelham *et al.* (*4*)
0–370	0–400	Vaughan *et al.* (*5*)
	Radioimmunoassay	
132–162	188–258	Arendt *et al.* (*6*)
*	188–481	Wetterberg *et al.* (*7*)
45–60	130–140	Arendt *et al.* (*8*)
11–32	42–110	Smith *et al.* (*9*)
7.5–20	40–100	Kennaway *et al.* (*3*)
	Gas chromatography–mass spectrometry	
25–40	125–440	Wilson *et al.* (*10*)
1.5–4.9	19.4–42.6	Lewy and Markey

*All samples were collected just before breakfast.

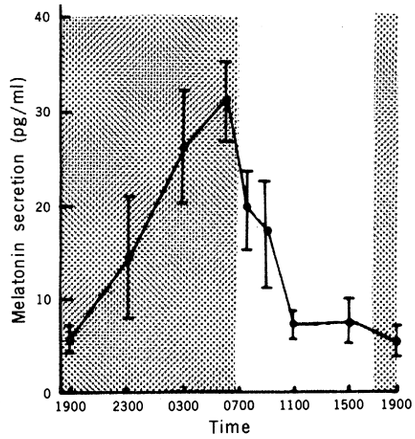

Fig. 5. Melatonin secretion, over a 24-hour period, in four normal subjects (two males and two females); the expected nighttime rise and daytime decrease are shown.

A typical standard curve is shown in Fig. 3, which is linear between 0.5 pg/ml and at least 200 pg/ml. The minimum detectable concentration is approximately 1 pg/ml (plasma), of which 200 to 500 femtograms was injected. When the chart paper is run at high speed (5 cm/min), the chromatographic peaks can be examined for symmetry and the absence of "shoulders" as an additional assurance of specificity. Typical signals from 50 pg of nondeuterated melatonin and 19 pg of tetradeuterated melatonin in a plasma sample are shown in Fig. 4. Variability within and between assays was 6 percent and 10 percent, respectively, for a pooled daytime plasma (approximately 10 pg/ml).

Analysis of plasma from two male and two female normal subjects is shown in Fig. 5. Daytime values were as low as 1.5 pg/ml, nighttime values were as high as 42.6 pg/ml, showing a 25-fold fluctuation. The timing of the highs and lows of melatonin levels with the dark-light cycle is consistent with previous reports. However, nighttime values are somewhat lower and daytime values are markedly lower than most previous reports (see Table 1), in which the dynamic range is considerably smaller. Values published a few years ago (Table 1) are considerably higher than those reported more recently; however, there appears to be a trend toward the levels found with negative CI GC-MS. Negative CI

MS may help to resolve controversies generated in the last few years regarding the exact amount and range of melatonin secretion in humans and regarding the nature of (i) pulsatile secretion, (ii) extrapineal sources (such as brain, peripheral nerve, harderian gland, retina, gastrointestinal tract), and (iii) urinary excretion.

Although the negative CI GC-MS technique is practicable for routine analyses of plasma (120 analyses per week), RIA's offer somewhat greater efficiency for analysis of larger numbers of samples. The RIA techniques do not require expensive equipment, and they provide a convenient means of assay that could be used for routine analyses; GC-MS could be reserved for validation of antiserum specificity and extraction procedures. In addition, the high sensitivity and selectivity attainable with negative CI GC-MS offers increased opportunity for detection and quantification of other trace organic compounds in a biological matrix at concentrations of parts per trillion.

ALFRED J. LEWY

Clinical Psychobiology Branch, National Institute of Mental Health, Bethesda, Maryland 20014

S. P. MARKEY

Laboratory of Clinical Science, National Institute of Mental Health

References and Notes

1. Pineal melatonin was first measured by bioassay; see A. B. Lerner and M. R. Wright [*Methods Biochem. Anal.* **8**, 295 (1960)], F. C. G. van de Veerdonk [*Nature (London)* **208**, 1324 (1965)], M. E. Tomatis and R. Orias [*Acta Physiol. Lat. Am.* **17**, 227 (1967)], and C. L. Ralph and H. J. Lynch [*Gen. Comp. Endocrinol.* **15**, 334 (1970)]. Fluorometric assays for pineal melatonin were developed by J. Axelrod and H. Weissbach [*J. Biol. Chem.* **236**, 211 (1961)], W. B. Quay [*Anal. Biochem.* **5**, 51 (1963)], and R. P. Maickel and F. P. Miller [*Adv. Pharmacol.* **6A**, 71 (1968)]. Pineal melatonin has also been measured by gas chromatography; see P. H. Degen, J. R. DoAmaral, J. D. Barchas [*Anal. Biochem.* **45**, 634 (1972)] and A. C. Greiner and S. C. Chan [*Science* **199**, 83 (1978)]. Radioimmunoassays for melatonin in tissues and species other than human plasma have been developed by L. J. Grota and G. M. Brown [*Can. J. Biochem.* **52**, 196 (1974)], L. Levine and L. J. Riceberg [*Res. Commun. Chem. Pathol. Pharmacol.* **10**, 693 (1975)], M. D. Rollag and G. D. Niswender [*Endocrinology* **98**, 482 (1976)], and R. J. Wurzburger, K. Kawashima, R. L. Miller, S. Spector [*Life Sci.* **18**, 867 (1976)]. GC-MS has been used to identify melatonin by Pelham *et al.* (*2*), F. Cattabeni, S. H. Koslow, E. Costa [*Science* **178**, 166 (1972)], I. Smith, P. E. Mullen, R. E. Silman, W. Snedden, B. W. Wilson [*Nature (London)* **260**, 718 (1976)], and Kennaway *et al.* (*3*). Melatonin has been measured in human urine by H. J. Lynch, R. J. Wurtman, M. A. Moskowitz, M. C. Archer, M. H. Ho [*Science* **187**, 169 (1975)], H. J. Lynch, Y. Ozaki, D. Shakal, R. J. Wurtman [*Int. J. Biometeorol.* **19**, 267 (1975)], and D. C. Jimerson, H. J. Lynch, R. M. Post, R. J. Wurtman, W. E. Bunney, Jr. [*Life Sci.* **20**, 1501 (1977)].
2. R. W. Pelham, C. L. Ralph, I. M. Campbell, *Biochem. Biophys. Res. Commun.* **46**, 1236 (1972).
3. D. J. Kennaway, R. G. Frith, G. Phillipou, C. D. Matthews, R. F. Seamark, *Endocrinology* **101**, 119 (1977).
4. R. W. Pelham, G. M. Vaughan, K. L. Sandock, M. K. Vaughan, *J. Clin. Endocrinol. Metab.* **37**, 341 (1973).
5. G. M. Vaughan, R. W. Pelham, S. F. Pang, L. L. Loughlin, K. M. Wilson, K. L. Sandock, M. K. Vaughan, S. H. Koslow, R. J. Reiter, *ibid.* **42**, 752 (1976).
6. J. Arendt, L. Paunier, P. C. Sizonenko, *ibid.* **40**, 347 (1975).
7. L. Wetterberg *et al.*, *ibid.* **42**, 185 (1976).
8. J. Arendt, L. Wetterberg, T. Heyden, P. C. Sizonenko, L. Paunier, *Horm. Res.* **8**, 65 (1977).
9. J. A. Smith, D. Padwick, T. J. X. Mee, K. P. Minneman, E. D. Bird, *Clin. Endocrinol.* **6**, 219 (1977).
10. B. W. Wilson, W. Snedden, R. E. Silman, I. Smith, P. Mullen, *Anal. Biochem.* **81**, 283 (1977).
11. D. F. Hunt, G. C. Stafford, Jr., F. W. Crow, J. W. Russell, *Anal. Chem.* **48**, 2098 (1976).
12. Melatonin (Sigma), acetonitrile (Fischer, pesticide grade), chloroform (Mallinckrodt, nanograde), boric acid (Fischer, certified A.C.S.), petroleum ether (Mallinckrodt, nanograde), benzene (Fischer, pesticide grade), pentafluoropropionic anhydride (Pierce), hexane (Burdick & Jackson, nonspectro grade), ethyl acetate (Fischer, certified A.C.S.), potassium chloride (Baker, Baker Analyzed), ammonium hydroxide (Mallinckrodt, analytical grade).
13. D. C. Fenimore, C. M. Davis, J. H. Whitford, C. A. Harrington, *Anal. Chem.* **48**, 2289 (1976).
14. G. J. Shaw, G. J. Wright, G. W. A. Milne, *Biomed. Mass Spectrom.* **4**, 348 (1977).
15. K. Blau, G. S. King, M. Sandler, *ibid.*, p. 232.
16. G. C. Stafford, Jr., paper FA2 presented at the 26th Annual Conference on Mass Spectrometry and Allied Topics, St. Louis, 28 May to 2 June 1977.
17. We thank Anthony P. Zavadil, Irwin J. Kopin, Frederick K. Goodwin, Thomas A. Wehr, Philip W. Gold, Donald McKenzie, and Pamela Greenlaw for their advice and assistance.

19 June 1978

Cellulose to Sugars: New Path Gives Quantitative Yield

Abstract. *Cellulosic residues that had been treated with a small amount of chemical solvent under room conditions were quantitatively saccharified on enzyme hydrolysis. This treatment can be used to obtain simple sugars for the production of alcohol and other chemicals.*

We have found a way to obtain quantitative yields of glucose from the alpha cellulose in agricultural residues. Cornstalks, bagasse, alfalfa, tall fescue, and orchard grass pretreated with an organic solvent give up to 99 percent glucose conversions on hydrolysis by cellulase enzymes from *Trichoderma reesei* (formerly *T. viride*).

The saccharification of cellulosics has been studied for 90 years (*1*, *2*). To date, cellulose hydrolysis has been accomplished by using either acids or enzymes (*2*–*5*). However, sulfuric acid promotes the formation of undesirable sugar side products (*2*). This problem does not exist when cellulose is hydrolyzed by the multicomponent enzyme system known as cellulase. This system hydrolyzes cellulose, a high polymer of glucose, to cellodextrins, which are water-soluble polymers with a degree of polymerization of < 6, and to glucose (*3*–*5*) without the formation of other, undesirable side products. Cellulase from *Trichoderma reesei* is of practical interest since recent pilot studies and the development of a hyperproducing mutant indicate that commercial production of this enzyme is feasible (*6*–*8*). A drawback to the use of cellulases has been their inability to rapidly and totally degrade native cellulose (*9*). The crystalline structure of cellulose and the lignin that physically seals the surrounding cellulose fibers make enzyme hydrolysis difficult by protecting the cellulose from contact with the enzyme.

To quickly and accurately quantify cellulose in various samples of cellulosic residues, we wished to develop an analysis that would involve (i) extraction of the cellulose from the sample, (ii) hydrolysis of the cellulose by either an enzyme or an acid, and (iii) calculation of the cellulose content from the hydrolysis kinetics. An extensive literature survey was undertaken to screen for cellulose solvents suitable for this purpose. Four major categories of solvents were uncovered: strong mineral acids (*10*); quaternary ammonium (Triton) bases (benzyltrimethylammonium hydroxides) (*11*); aprotic solvents (dimethyl sulfoxide–paraformaldehyde, nitrosylics, sulfur oxides, and oxychlorides) (*12*); and metal complexes such as cupriethylene diamine, cadoxen, and zincoxen (*13*). Although these solvents have been used for viscometric studies of cellulose (*14*), the combination of solvent pretreatment and enzyme hydrolysis appeared to be a new concept.

A combination of intuition and trial and error resulted in the choice of cadoxen as a solvent. Cadoxen is a colorless, odorless solvent made by dissolving 5 percent cadmium oxide in 28 percent aqueous ethylenediamine. Procedures for making the solvent and its properties have been described (*15*–*17*).

The application of cadoxen in an analytical procedure worked well. Cellulose from various samples was extracted in excess cadoxen and hydrolyzed. The hydrolysis gave quantitative conversion of cellulose to sugars. Thus, the cellulose content could be directly calculated from the quantity of sugar formed without using complicated kinetic equations.

Success with the analytical procedure led to the development of a process that should be feasible on a commercial scale. In this process, particles of cellulosics in the size range 0.5 to 2 mm (or larger) are placed in small amounts of cadoxen at room temperature and let

0036-8075/78/0825-0743$00.50/0