Journal of Pharmaceutical & Biomedical Analysis
Vol. 5, No. 2, pp. 131–140, 1987
Printed in Great Britain

0731–7085/87 $3.00 + 0.00

Characterization of digoxin and related cardiac glycosides by fast atom bombardment mass spectrometry*

J. R. JOCELYN PARÉ†[1], PIERRE LAFONTAINE[1], JACQUELINE BÉLANGER†[2], WING-WAH SY[2], NICOLE JORDAN[2] and JACK C. K. LOO[2]

[1] *Chemistry and Biology Research Institute, Agriculture Canada, K.W. Neatby Bldg., Ottawa, Ontario K1A 0C6, Canada*

[2] *Bureau of Drug Research, Health Protection Branch, Health and Welfare Canada, Banting Research Center, Ottawa, Ontario K1A 0L2, Canada*

Abstract: The potential of using fast atom bombardment mass spectrometry for the detection and the characterization of digoxin and a series of related cardenolide analogues was investigated. The spectra were dependent upon the type of support-matrix in which they were recorded; thioglycerol proved to be satisfactory for the characterization of digoxin and allowed for its detection in human urine extract spiked with *ca* 11 ng ml^{-1}.

Keywords: *FAB–MS; cardiac glycosides; thioglycerol; digoxin.*

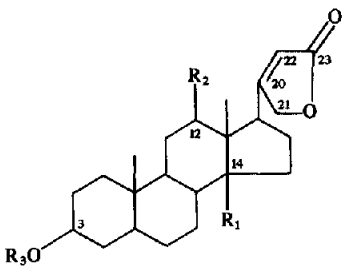
Introduction

The cardiac glycosides are important therapeutic agents used in the treatment of congestive heart failures and certain arrythmias. Their uniqueness resides in their ability to increase both the efficiency and the contractility of the heart muscle. Digitoxin (**1**) and digoxin (**2**) are the most widely used *digitalis* glycosides in clinical applications. Both of them possess a relatively narrow therapeutic index and symptoms of toxicity can be confused with the actual state of the disease being treated.

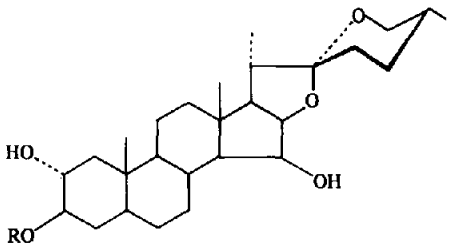
These molecules have long resisted structural elucidation work by electron impact (EI–MS) [1, 2], although some success was obtained by other mass spectral techniques such as field desorption (FDMS) [3, 4], laser desorption (LDMS) [5, 6], ammonia chemical ionization (CIMS) [7], flash ionization MS [8] and more recently by DCI (OH^-) [9, 10], where $[M-H]^-$ ions are present as base peaks. The development of fast atom bombardment mass spectrometry (FABMS) [11] for the analysis of polar, thermally labile glycosides such as these [12–16] prompted us to explore further the

*C.B.R.I. Contribution No. 1528. Presented in part to the 45th Int. Congress of Pharm. Sci. of Fed. Int. Pharm., Montréal, QC, Canada, September 1985.

†Authors to whom all correspondence should be addressed. Permanent address: Centre de Recherches Alimentaires de Saint-Hyacinthe, 3600, Casavant West, Saint-Hyacinthe, Quebec J2S 8E3, Canada.



Compound	R_1	R_2	R_3
Digitoxin **(1)**	OH	H	2,6 - Dideoxy - ribohexose (3 moles)
Digoxin **(2)**	OH	OH	2,6 - Dideoxy - ribohexose (3 moles)
Dihydrodigoxin **(3)** (No Δ 20,22)	OH	OH	2,6 - Dideoxy - ribohexose (3 moles)
Acetodigoxin **(4)**			
Digitoxigenin **(5)**	OH	H	H
Digoxigenin **(6)**	OH	OH	H



Compound	R
Digitonin **(7)**	D - galactose (2 moles)
	D - glucose (2 moles)
	D - xylose (1 mole)

application of FABMS to the detection and the characterization of a series of cardenolide analogues. A previous report [15] suggested that PEG was a more suitable matrix under negative ion FAB conditions, although no definitive conclusion can be reached from that paper. Other reports [17, 18] also discussed the advantages of negative ion detection over positive ion detection of cardenolides under various ionization conditions. In this study, however, several different matrices were used very successfully under positive ion FAB conditions and thioglycerol proved to be the matrix that allowed the best sensitivity. We now report that positive ion FABMS was useful in characterizing these cardiac glycosides and in detecting them in human urine at levels comparable to those reported by Levsen *et al.* [19] by on-line liquid chromatographic/chloride attachment mass spectra. The latter authors suggested that "Further improvements are conceivable if FABMS were used as the ionization mode. However, preliminary results demonstrated that . . . the sensitivity was poor". In this report we circumvent these allegations by optimizing the support-matrix system.

Experimental

Mass spectrometry

Digitoxin (USP Reference Standard) (**1**), digoxin (Sigma and USP standard) (**2**), dihydrodigoxin (Boehringer) (**3**), acetylated digoxin (obtained by direct acetylation of digoxin) (**4**), digitoxigenin (Sigma) (**5**), digoxigenin (Sigma) (**6**), digitonin (Sigma) (**7**), and "gitalin" (Sigma) were used as obtained. The spectra were recorded on a Finnigan MAT 312 reversed geometry double focussing mass spectrometer mounted with a saddle field atom gun (Ion Tech.) and coupled to an INCOS data system. High purity research grade Xenon (99.995%, Matheson) was used as the bombardment gas (at 8 kV) and the resulting positive ions were extracted (3 kV) into the mass analyser. The scan ranged between 110–1350 daltons and was performed exponentially in 10 s (electron multiplier set at 2.2 kV, resolution of 1500). The samples were introduced into the ion source as solutions, or dispersions, in glycerol, thioglycerol, polyethylene glycol-200 (PEG-200), diethanolamine, glycerol–NaCl mixture, glycerol–NaCl–NH_4OH mixture, glycerol–ethanolamine mixture, thioglycerol–diethanolamine mixture, PEG-200–NH_4OH mixture and diethanolamine–water mixture. All of these reagents used as matrices were obtained commercially and were not subjected to any further purification. A standard solution of digoxin containing *ca* 100 ng μl^{-1} was prepared by dissolving *ca* 1.0 mg of pure USP standard digoxin into 10 ml of glass-distilled ethyl acetate. That standard solution was then used to deliver the microsamples of digoxin onto the FAB target in the analyses attempting to establish a limit of detection and in the analyses involving the detection of digoxin in spiked human urine samples.

Purification of urine blank

Urine blank (30 ml) was purified according to a modified method of Loo *et al.* [20]. The modification includes loading 3 ml of urine onto a CLIN-ELUT (AnalytiChem. International, USA) and washing it with 12 ml of hexane. The 12 ml of methyl *t*-butyl ether was used to elute the applied sample. The collected sample was chromatographed by HPLC [cf. ref. 20] and the eluent corresponding to the retention time of digoxin was collected. The entire procedure was repeated 10 times and the eluents combined. One blank sample was subjected to MS analysis and a second portion was spiked with 200 ng of digoxin prior to MS analysis. Total recovery of extraction was 60% (as determined by using radio-labelled digoxin); the spiked sample therefore, corresponded to *ca* 11 ng ml^{-1} of urine.

Results and Discussion

All the spectra recorded in glycerol were informative in terms of molecular weight determination with the exception of digoxin and its two derivatives, acetylated digoxin and dihydrodigoxin. The spectra of these compounds were recorded in thioglycerol (digoxin and dihydrodigoxin) or in a thioglycerol–diethanolamine mixture (acetylated digoxin) whichever proved to be more informative. From an analytical standpoint, digoxin was the compound of prime interest and received more attention; thus spectra were recorded in 11 different matrices in order to determine the nature of the most suitable matrix system that could be used for routine digoxin analysis. Table 1 presents the list of compounds analysed along with the matrices in which spectra were recorded with some relevant comments on each.

Table 1
Information* obtained from FAB spectra of cardenolide analogues recorded in various matrices

Compound	Matrix	Comments
Digoxigenin (6)	Gly	$[M + H]^+$, small loss of H_2O $[H + H + Gly]^+$, almost no fragmentation observed
Digitoxigenin (5)	Gly–EA	$[M + H]^+$, so significant loss of H_2O, almost no fragmentation observed
	Gly–EA	$[M + H]^+$, $[M + EA + H]^+$, some $[M + Gly + H]^+$, some loss of H_2O, no fragmentation observed
Dihydrodigoxin (3)	Gly	no structural/molecular weight information
	Tgly	$[M + Na]^+$, weak $[M + H]^+$ weak $[M + H - H_2O]^+$, full sugar sequence was deduced from fragmentations observed
	Tgly–DEA	$[M + Na]^+$, $[M + DEA + H]^+$ no loss of H_2O, no fragmentation observed
Digoxin (2)	Gly	no structural/molecular weight information observed (Fig. 2a)
	Gly–EA	weak $[M + EA + H]^+$, no structural information
	Gly–NaCl	$[M + Na]^+$, no other structural information deduced
	Gly–NaCl–NH_4OH	only background was detected
	Gly–Tgly	as per Tgly, but apparent overall loss in sensitivity
	Tgly	$[M + H]^+$, no loss of H_2O from molecular ion, complete sugar sequence was deduced from fragmentation observed (Fig. 2b)
	Tgly–DEA	$[M + DEA + H]^+$, no loss of H_2O but no sequencing information
Digoxin (2)	DEA	$[M + DEA + H]^+$, no loss of H_2O, no sequencing information
	DEA–H_2O	$M + [DEA + H]^+$, no loss of H_2O, no sequencing information
	PEG-200	only background was detected
	PEG-200–NH_4OH	only background was detected

Table 1
(continued)

Compound	Matrix	Comments
Acetylated digoxin (4)	Gly	only background was detected
	Gly–EA	weak $[M + EA + H]^+$, trace of non-peracetylated material was detected, no sequence information
	Tgly	only background was detected
	Tgly–DEA	$[M + DEA + H]^+$, trace of non-peracetylated material was detected
Digitoxin (1)	Gly	$[M + H]^+$, $[M + Gly + H]^+$, little loss of H_2O, completed sugar sequence was deduced from recorded fragmentations (Fig. 1)
	Gly–EA	$[M + EA + H]^+$, no loss of H_2O, no other structural information
	Tgly	strong $[M + H]^+$, no loss of H_2O, complete sugar sequence was deduced from recorded fragmentations
	Tgly–DEA	$[M + DEA + H]^+$, no other structural information
Digitonin (7)	Gly	strong $[M + H]^+$, no loss of H_2O, complete sugar sequence was deduced from recorded fragmentations
Gitalin	Gly	$[M + H]^+$ observed for the 3 major constituents plus a fourth one, minor loss of H_2O, complete sugar sequence was possible when complemented with data from Gly–EA spectrum below, some noise
	Gly–EA	$[M + EA + H]^+$ for the 3 main constituents plus another one apparently different from the fourth one seen in Gly (above), no loss of H_2O, complete sugar sequence was possible when complemented with data from Gly spectrum above

*Complete spectral data are available from the authors (JRJP) upon request.

Gitalin is a mixture of cardiac glycosides containing relatively constant proportions of digitoxin, gitoxin and gitaloxin and smaller amounts of several other glycosides and genins, together with minute amounts of other substances [see ref. 11].

Gly = glycerol,
Tgly = Thioglycerol,
EA = ethanolamine,
DEA = diethanolamine,
PEG-200 = polyethylene glycol (average mol. wt. of 200).

It is interesting to note the marked difference in behaviour between digitoxin and digoxin. Digitoxin produces an excellent spectrum in glycerol that does not show any significant decomposition, yields a good $[M + H]^+$ ion (*m/z* 765) and also allows for the complete sequence of the carbohydrate residues to be established (Fig. 1). In contrast, the spectrum of digoxin in glycerol (Fig. 2a) does not show any feature of structural importance. When recorded in thioglycerol, however (Fig. 2b), the situation changed to one that is structurally useful (digitoxin offers the same sequencing information when recorded in thioglycerol). A similar difference in behaviour had been reported by other workers during their evaluation of CIMS as an analytical tool for these cardiac glycosides [7]. In contrast, no difference was found between the DCI (OH^-) spectra of digitoxin and digoxin apart from a shift in *m/z* by 16 a.m.u. [10]. We have recently presented an explanation for the better suitability of thioglycerol over glycerol [21] that seems to be applicable in this study as well.

With respect to the application of FABMS to the analysis of cardenolide analogues, it was possible to record a good spectrum of a 100 ng sample of digoxin in thioglycerol. Unfortunately, thioglycerol alone is difficult to use as the pressure in the ion source rises fairly high (*ca* 10^{-4} mbar). To circumvent that problem, we immersed the FAB target containing a 10 ng sample of digoxin in 2 μl of thioglycerol into liquid nitrogen prior to recording the spectrum. We were successful at recording the $[M + H]^+$ peak with a S/N >5 but were unable to get consistently reproducible spectra as we had no control on the temperature change occurring in the ion source. The present detection limit is already

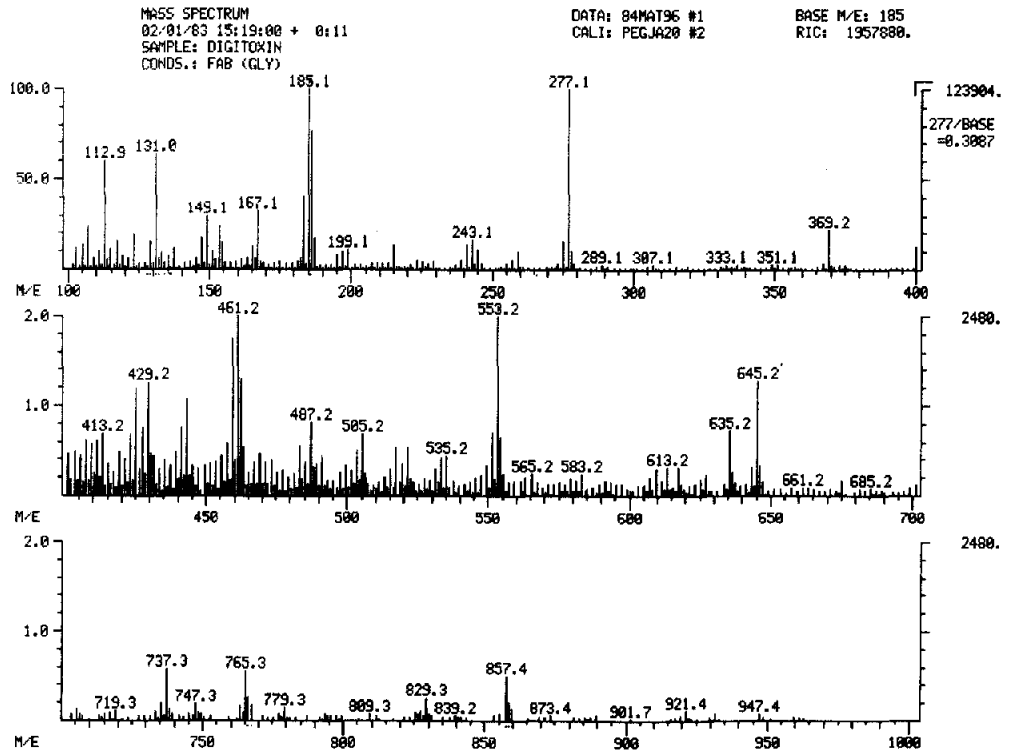


Figure 1
FAB mass spectrum of digitoxin (1) recorded in glycerol.

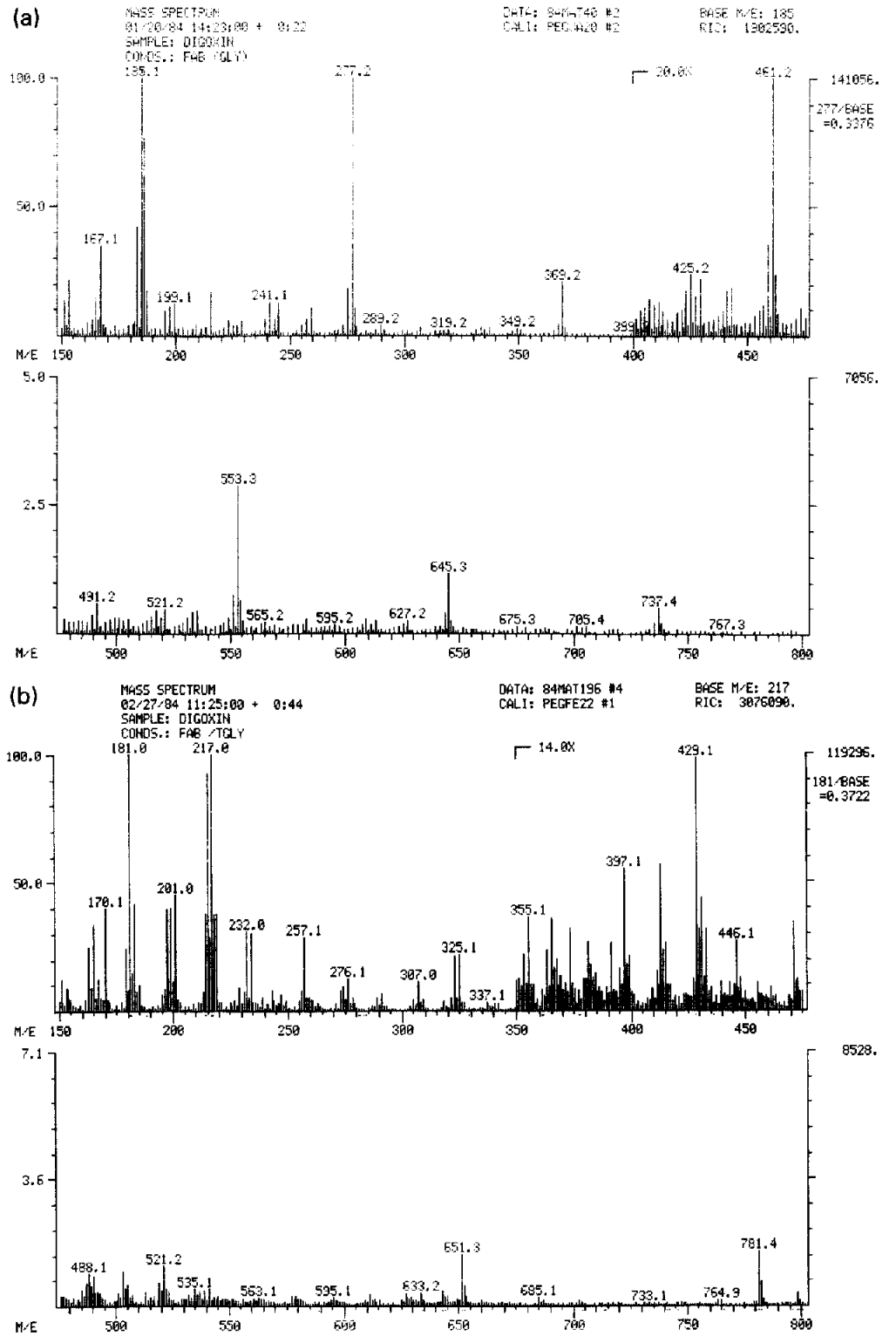


Figure 2
FAB mass spectra of digoxin (**2**) recorded in glycerol (a) and in thioglycerol (b). Sample size for both cases: 1 μg in 2 μl.

comparable to that of other workers [19] despite this experimental drawback that should be controllable through future refinements of the sample introduction method. It is interesting to note that the overall appearance of the spectra, exclusion made of the matrix-related peaks, did not change with the lowering of the quantities analysed but for the intensity of all the ions. This phenomenon differs from that observed under LC–MS conditions [19, 22] and that reported for digitoxin under DCI $(OH)^-$ conditions [23].

Figure 3 shows another example of an analytical application of FAB for the screening of cardiac glycosides in original samples. Part a depicts a blank human urine sample, part b is the spectrum of the same blank human urine extract sample spiked with 200 ng of digoxin (initial concentration of 11 ng ml^{-1} of urine) and, part c shows the spectrum of a 200 ng pure USP standard digoxin sample. All of these spectra were recorded in a glycerol–thioglycerol mixture to allow for a longer recording time despite the inherent loss of sensitivity over the use of pure thioglycerol; the intention here was to show the ability of the technique to detect digoxin despite the background arising from the other compounds present in the urine.

Unlike a previous report [19], the data presented here suggest a rather bright future for FABMS as an analytical screening technique for the presence of cardiac glycosides, more specifically of digoxin, in urine samples. The technique offers structural

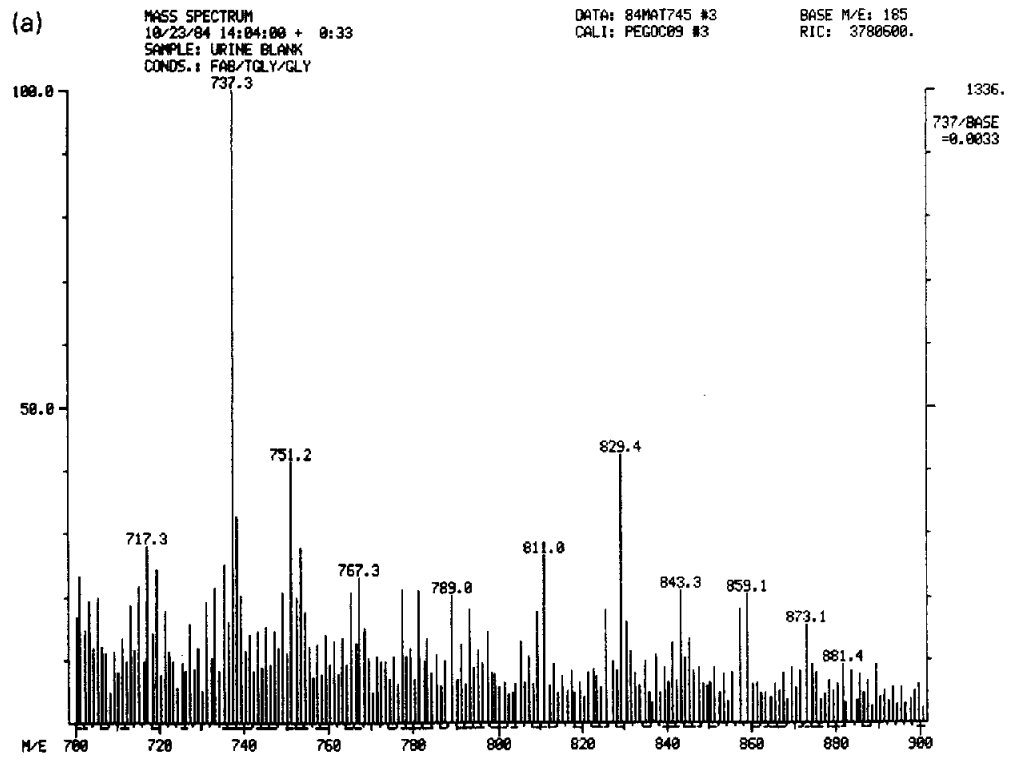
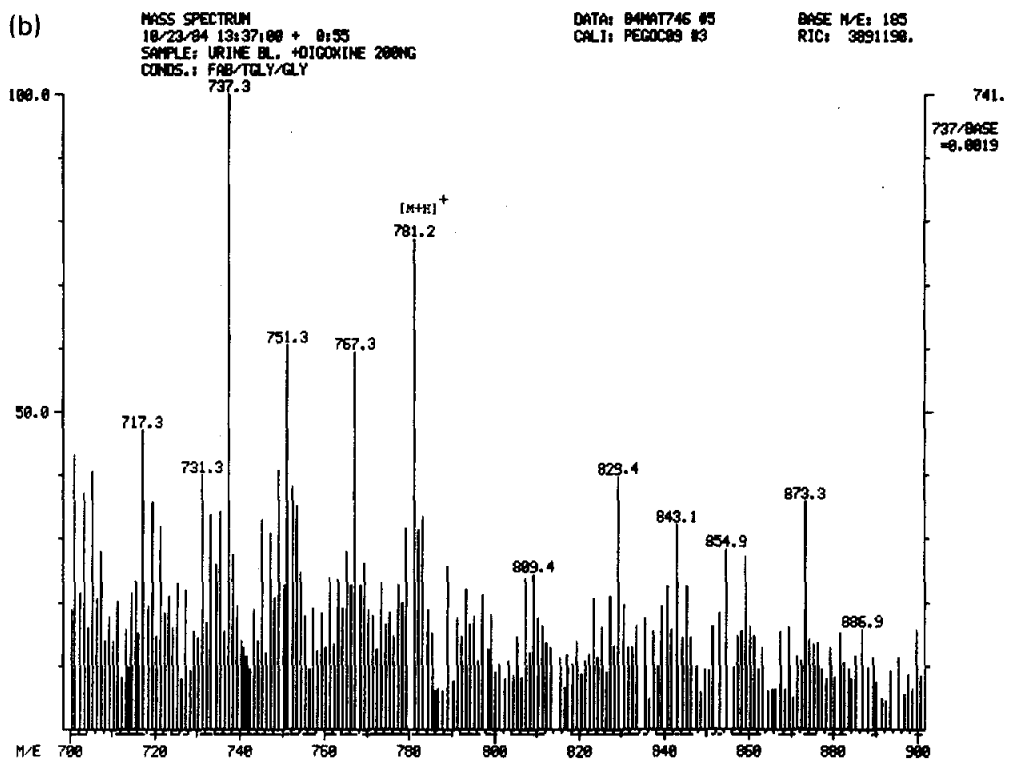
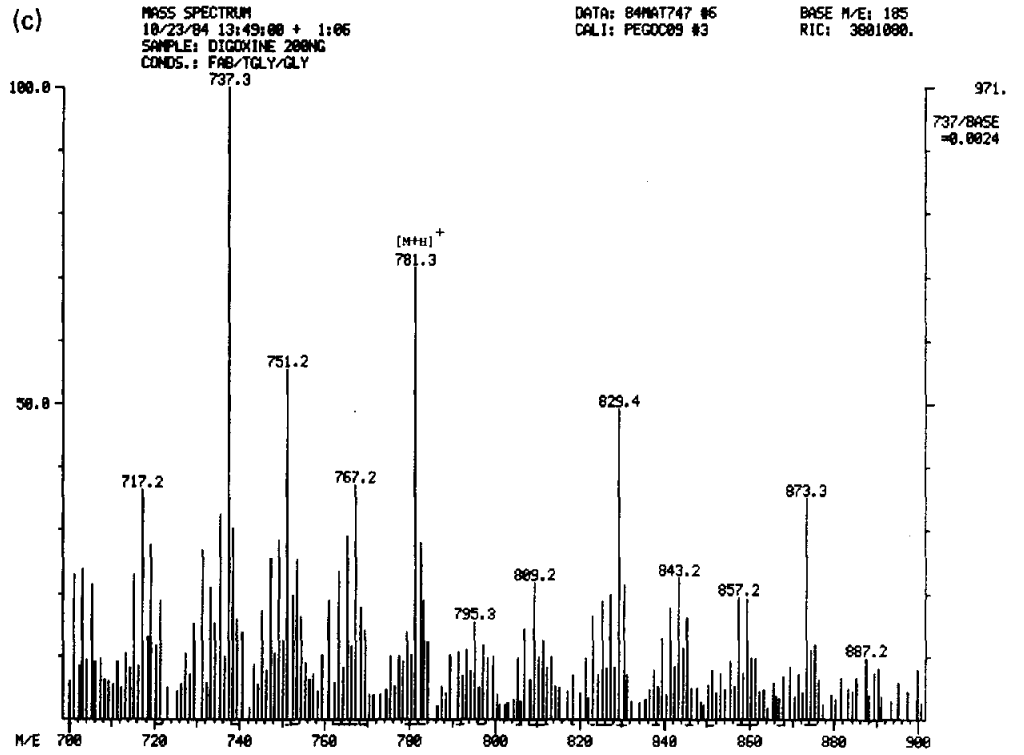


Figure 3
(a) Typical FAB mass spectrum obtained from a human urine blank; (b) FAB mass spectrum of the same human urine blank as above spiked with 200 ng of digoxin (2), and (c) FAB mass spectrum of a 200 ng sample of digoxin (2). All of the above spectra were recorded in a glycerol–thioglycerol mixture. Sample size in all cases 2 μl.


(b)
MASS SPECTRUM
10/23/84 13:37:00 + 0:55
SAMPLE: URINE BL. +DIGOXINE 200NG
CONDS.: FAB/TGLY/GLY
DATA: 84MAT746 #5
CALI: PEGDC09 #3
BASE M/E: 185
RIC: 3891190.
741.
737/BASE
=0.0019
737.3
[M+H]+
781.2
751.3
767.3
717.3
731.3
829.4
843.1
854.9
873.3
809.4
886.9
100.0
50.0
M/E 700 720 740 760 780 800 820 840 860 880 900


(c)
MASS SPECTRUM
10/23/84 13:49:00 + 1:06
SAMPLE: DIGOXINE 200NG
CONDS.: FAB/TGLY/GLY
DATA: 84MAT747 #6
CALI: PEGDC09 #3
BASE M/E: 185
RIC: 3801000.
971.
737/BASE
=0.0024
737.3
[M+H]+
781.3
751.2
829.4
717.2
767.2
873.3
809.2
843.2
857.2
795.3
887.2
100.0
50.0
M/E 700 720 740 760 780 800 820 840 860 880 900

information advantages over that of DCI (OH^-) [10] and appears to be as sensitive or more sensitive than previously reported ones that invariably made use of negative ion detection [15–19]. Although FAB was previously shown to be amenable to reliable quantitative studies [24, 25] and to high resolution analyses [26] that should render the technique even more powerful, no definitive report has been presented to date to establish the former unequivocally at the levels of detection of interest for these cardiac glycosides.

Acknowledgements: We thank the Natural Sciences and Engineering Research Council (Canada) for the award of a Visiting Fellowship in Biotechnology to J.R.J.P.

References

[1] P. Brown, F. R. Brüschweiler, G. R. Pettit and T. Reichstein, *J. Am. Chem. Soc.* **92**, 4470 (1970).
[2] H. Greenwood, W. Snedden, R. P. Hayward and J. Landon, *Clin. Chim. Acta* **62**, 213 (1975).
[3] R.-R. Schulten, T. Komori and T. Kawasaki, *Tetrahedron* **33**, 2596 (1977).
[4] H.-R. Schulten, T. Komori, T. Nohara, R. Higuchi and T. Kawasaki, *Tetrahedron* **34**, 1003 (1978).
[5] M. A. Posthumus, P. G. Kistermaker, H. L. C. Meuzelaar and M. C. Ten Noever de Brauw, *Anal. Chem.* **50**, 985 (1978).
[6] R. J. Cotter, *Anal. Chem.* **56**, 985 (1978).
[7] J. Vine, L. Brown, J. Boutagy, R. Thomas and D. Nelson, *Biomed. Mass Spectrom.* **6**, 415 (1979).
[8] T. D. Lee, W. R. Anderson, Jr., G. D. Daves, Jr., E. Haslam, A. G. Boettger and C. E. Giffin, *Int. J. Spectrosc.* **1**, 110 (1982).
[9] A. P. Bruins, *Adv. Mass Spectrom.* **8**, 246 (1980).
[10] A. P. Bruins, *Int. J. Mass Spectrom. Ion Phys.* **48**, 185 (1983).
[11] M. Barber, R. S. Bordoli, R. D. Sedgwick and A. N. Tyler, *J. Chem. Soc. Chem. Commun.* 325 (1981).
[12] J. R. J. Paré, Ph.D. thesis, Chapter 2, Carleton University, Ottawa, Ontario, Canada (1984).
[13] J. Bélanger, Ph.D. thesis, Carleton University, Ottawa, Ontario, Canada (1984).
[14] F.-X. Garneau, J.-L. Simard, O. Harvey, J. W. ApSimon and M. Girard, *Can. J. Chem.* **61**, 1465 (1983).
[15] M. E. Rose, M. P. Veares, I. A. S. Lewis and J. Goad, *Biochem. Soc. Trans.* **11**, 602 (1983).
[16] M. Adinolfi, L. Mangoni, G. Marino, M. Parrilli and R. Self, *Biomed. Mass Spectrom.* **11**, 310 (1984).
[17] D. J. Dixon, *Analysis* **10**, 343 (1982).
[18] M. von Ardenne, R. Tuemmler, E. Weiss and T. Reichstein, *Helv. Chim. Acta* **47**, 1032 (1984).
[19] K. Levsen, K. H. Schäfer and P. Dobberstein, *Biomed. Mass Spectrom.* **11**, 308 (1984).
[20] J. C. K. Loo, I. J. McGilveray and N. Jordan, *Res. Commun. Chem. Pathol. Pharmacol.* **17**, 497 (1977).
[21] J. Bélanger, B. A. Lodge, J. R. J. Paré and P. Lafontaine, *J. Pharm. Biomed. Anal.* **3**, 81 (1985).
[22] D. E. Games, M. A. McDowall, K. Levsen, K. H. Schäfer, P. Dobberstein and J. L. Gower, *Biomed. Mass Spectrom.* **11**, 87 (1984).
[23] A. P. Bruins, *Anal. Chem.* **52**, 605 (1980).
[24] A. A. Graluska and G. H. Morrison, *Anal. Chem.* **56**, 74 (1984).
[25] K. L. Clay, L. Wählin and R. C. Murphy, *Biomed. Mass Spectrom.* **10**, 489 (1983).
[26] K. L. Clay and R. C. Murphy, *Int. J. Mass Spectrom. Ion Phys.* **53**, 327 (1983).