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High-performance liquid chromatography–ionspray mass spectrometry for the specific determination of digoxin and some related cardiac glycosides in human plasma

A. Tracqui*, P. Kintz, B. Ludes, P. Mangin

Institut de Médecine Légale, 11 Rue Humann, 67085 Strasbourg Cedex, France

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Abstract

An original method based upon high-performance liquid chromatography coupled to ionspray mass spectrometry (HPLC–ISP-MS) has been developed for the identification and quantification in plasma of several cardiac glycosides, namely digoxin, digitoxin, lanatoside C and acetyldigitoxin. After single-step liquid–liquid extraction by chloroform–2-propanol (95:5, v/v) at pH 9.5 using oleandrin as an internal standard, solutes are separated on a 4 μm NovaPak C_{18} (Waters) column (150×2.0 mm, I.D.), using a gradient of acetonitrile–2 m*M* NH_4COOH, pH 3 buffer (flow-rate 200 μl/min, post-column split 1:3). Detection is done by a Perkin-Elmer Sciex API-100 mass analyzer equipped with an ISP interface. In most instances the major ion observed is not $[M+H]^+$ as expected, but $[M+NH_4]^+$. The mean retention times (min) are: lanatoside C, 5.74; digoxin, 6.00; digitoxin, 8.08, oleandrin, 8.30, acetyldigitoxin, 8.66 and 9.01 (isomers α and β, respectively). The lower limits of detection in single ion monitoring mode range from 0.15 ng/ml (α- and β-acetyldigitoxin) to 0.60 ng/ml (lanatoside C), making the method less sensitive than radioimmunoassay, whereas it is much more specific.

Keywords: Digoxin; Digitoxin; Lanatoside; Acetyldigitoxin

1. Introduction

Cardiotonic glycosides (CGs) have been used for more than 200 years for treating congestive heart failure and some other cardiac diseases, notably atrial arrhythmias [1]. Digoxin is by far the most commonly prescribed CG over the world, although the longer-acting digitoxin is also popular in Europe [2]; in France, these two compounds account for 70% and 20% of CG sales, respectively [3]. More rarely employed CGs include α-acetyldigitoxin, acetyldigoxin, lanatoside C, β-methyldigoxin, ouabain, proscillaridin and strophantin-K [1,2]. All these drugs have the same action on cardiac parameters, but differ strongly in their respective pharmacokinetics which mainly reflect their polarity [4].

The determination of digoxin and related CGs in biological fluids is useful for therapeutic drug monitoring, because these compounds are among the cardiovascular drugs with the narrowest toxic/therapeutic margins [3,4]. It is generally performed by radioimmunoassay (RIA), a highly sensitive technique well adapted to the low plasmatic concentrations at which CGs are active. Unfortunately RIA only offers a group-selectivity: the common digoxin kits exhibit marked cross-reactivity with many of the

*Corresponding author.

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numerous digoxin metabolites (active or inactive), as well as with other CGs (e.g. 0.6 to 25% with digitoxin) [5,6]. In addition, authentic false positives may result from endogenous, digoxin-like immunoreactive substances that have been reported to occur in the biofluids of pregnant women, newborn infants, or subjects suffering from renal failure or liver dysfunction [5,7–10]. A number of sophisticated techniques have been proposed in order to minimize or suppress these interferences, including RIA following either ultrafiltration [11] or solvent extraction [12] of the serum, combined thin-layer chromatography–RIA [13] or high-performance liquid chromatography (HPLC)–RIA [14,15], as well as HPLC with formation of a fluorescent derivative [16]. In some circumstances however (e.g. forensic situations), the unequivocal identification of a drug remains of prime necessity. Since the nonvolatile and thermolabile CGs are not amenable to gas chromatography–mass spectrometry (GC–MS, the technique routinely used in most instances to bring this conclusive evidence), we proceeded to develop an alternative procedure based upon HPLC coupled to ionspray MS (HPLC–ISP-MS); this paper presents its performances and limitations.

2. Experimental

2.1. Materials

Digoxin (MW=780.9), digitoxin (MW=764.9), ouabain (MW=584.6), lanatoside C (MW=985.1), acetyldigitoxin (MW=807.0) as well as the internal standard (I.S.) oleandrin (MW=576.7) were all from Sigma. Methanol and acetonitrile were HPLC grade (Merck). Concentrated (99–100%) formic acid (HCOOH) was Normatom grade (Prolabo, France). Chloroform, 2-propanol, ammonium chloride (NH_4Cl), concentrated (ca. 20%) ammonia solution (NH_4OH) and ammonium formate (NH_4COOH) were analytical grade and purchased from Merck, Fluka and Prolabo.

Stock solutions (100.0 μg/ml) of digoxin, digitoxin, ouabain, lanatoside C, acetyldigitoxin and oleandrin were prepared in methanol and stored at +4°C in the dark, where they were found to be stable for at least one month. Work solutions were obtained just before use by appropriate dilutions in 1.5 ml Eppendorff-type plastic microtubes made opaque using aluminium foils. The pH 9.5 buffer was prepared using a saturated (25–28%, w/v) NH_4Cl solution, 25% diluted with deionized water then adjusted to the desired pH by appropriate addition of conc. NH_4OH. The pH 3.0 buffer was prepared using a 2 m*M* NH_4COOH solution (126.2 μg/ml) adjusted to the desired pH by appropriate addition of conc. HCOOH.

A 10^{-4} *M* solution of high MW poly(propylene glycol)s (PPGs) in water–methanol (50:50, v/v, +2 m*M* ammonium acetate+0.1% HCOOH+0.1% acetonitrile), provided by Perkin-Elmer Sciex (Foster City, CA, USA), was used for mass analyzer tuning.

2.2. Chromatography

A 20 ml dual-syringe HPLC pump (Applied Biosystems Model 140B) was employed to deliver the pulse-free, low flow-rates required by the ISP interface. Samples were manually injected using a 100 μl gastight syringe (Hamilton Model 1710) and a Rheodyne Model 8125 low-dispersion valve equipped with a home-made, 5.0 μl PEEK loop (0.005″ I.D.). Applications entailing continuous infusion of a definite analyte (e.g. MS tuning or spectrum determinations) were carried out using a precision, single-syringe low-pressure infusion pump (Harvard Apparatus Model 11).

The HPLC separations were performed on a 4 μm NovaPak C_{18} (Waters) column (150×2.0 mm, I.D.), operated at ambient temperature and protected by a 5 μm Opti-Guard C_{18} (Interchim, France) fingertight guard cartridge (15×1.0 mm, I.D.). Each 10 min chromatographic run was carried out with a binary mobile phase of acetonitrile–2 m*M* NH_4COOH, pH 3.0 buffer, using the following gradient: acetonitrile, 20% at t_0 to 38% at 5.0 min; up to 65% at 6.0 min; up to 70% at 8.5 min then isocratic to 9.5 min; down to 20% at 10 min. The flow-rate was 200 μl/min (operating pressure in the range 4.7 to 8.1 MPa) with a post-column split (a zero-dead volume tee with two outlets of unequal length) of 1:3, to reduce the flow-rate infused into the ISP to 50 μl/min. An equilibration time of 5 min at 20% acetonitrile was allowed between two successive runs. Before use, the components of the mobile phase were degassed

and filtered through 0.45-μm filters (Durapore GVWP 047, Millipore) with a Pyrex filter holder (Millipore); at the end of each chromatographic session, the column was unplugged from the detector, then thoroughly rinsed with acetonitrile–deionized water (80:20 for 30 min, then 50:50 for 3 h) at a flow-rate of 200 μl/min.

2.3. Mass spectrometry

MS detection was carried out using a Perkin-Elmer Sciex API-100 single-quadrupole instrument. The system was monitored by an Apple Macintosh PowerPC 8100/80 computer equipped with the softwares LC2Tune v. 1.1, MultiView v. 1.1, and MacQuan v. 1.4 (Perkin-Elmer Sciex) for instrument control and data acquisition, data reprocessing, and solute quantification, respectively. Nitrogen (purity grade U, i.e. 99.95%, 40 psi) was employed as the nebulizing gas (flow-rate 1.16 l/min). The instrument was operated in the positive ionization mode with a voltage of +4.5 kV applied to the sprayer during all experiments. Ions generated in the ion source were sampled into the mass analyzer by passing successively through a 25 μm I.D. orifice (OR) at the rear end of the atmospheric chamber, an intermediate pressure region (ca. 2.2 Torr) and a conical skimmer (held at ground potential) encircled by a focusing ring maintained at +400 V. To prevent solvent vapours and contaminants from entering the vacuum chamber, the area in front of the OR was continuously flushed with a "curtain gas" (N_2 purity U, 40 psi) at a flow-rate of 1.08 l/min during all experiments, and 0.14 l/min when the instrument was set in overnight standby. The vacuum chamber housing the ion transfer and focalisation quadrupole (Q0), the filtration quadrupole (Q1), the ion optics and the electron multiplier (EM) was divided into two regions (Q0 and Q1) respectively maintained at about 8.10^{-3} and 2.10^{-5} Torr by a staged combination of two built-in turbomolecular pumps (50 and 150 l/s) and an external, rotary-vane backing pump (Trivac Model D8A, Leybold, Canada).

The system was weekly tuned by using a continuous infusion at 5 μl/min of the standard mixture of PPGs, and monitoring the ions at m/z 59, 175, 616, 907, 1255, 1545, 1836 and 2010 for mass calibration. lens optimization and peak width adjustments. For routine determinations, the main instrument settings were: OR, +20 V; Q0, −10 V; IQ1 (lens), −12 V; ST (lens), −15 V; Q1, −16 V; EM, +2600 V. MS data were collected as either total ion chromatograms (TIC) by monitoring the signal over a large, continuous mass range, or in the single ion monitoring (SIM) mode by focusing the detector on a sole mass, or on a discrete list of masses, respectively.

2.4. Extraction procedure

To 4.0 ml of plasma in 15 ml Pyrex centrifuge tubes were added 40 μl of a 1.0 μg/ml methanolic solution of oleandrin (I.S.), 1.0 ml of the NH_4Cl, pH 9.5 buffer, and 5.0 ml of chloroform–2-propanol (95:5, v/v). This mixture was gently shaken on a horizontal agitator for 10 min, then centrifuged at 3500 *g* for 10 min. The lower organic phase was removed into 5 ml borosilicate glass tubes and evaporated to dryness at 45°C in a rotary evaporator (Speed Vac concentrator, Model A 290, Savant Instruments); after adding 25 μl of methanol–2 m*M* NH_4COOH, pH 3.0 buffer (20:80, v/v) and vortexing (10 s), the extract was transferred into 1.5 ml Eppendorff-type plastic microtubes that were centrifuged at 10 000 *g* for 5 min. A 15 μl volume of the supernatant was then pipetted, from which 5 μl was injected onto the column at each chromatographic run.

3. Results and discussion

Fig. 1 shows the full-scan, background-subtracted ISP mass spectra of digoxin, digitoxin, ouabain, lanatoside C, acetyldigitoxin, and oleandrin. This was recorded from a continuous, 5 μl/min syringe infusion of a 10.0 μg/ml solution of each CG, corresponding to a decimal dilution of the 100.0 μg/ml stock solution into a mixture of acetonitrile–2 m*M* NH_4COOH, pH 3.0 buffer (50:50, v/v). The major ions observed for each compound are listed in Table 1. Although protonation generally represents the main ionization process when using atmospheric pressure HPLC–MS interfaces [17–20], cluster formation is also a typical feature of mass spectra generated by these devices, and may sometimes

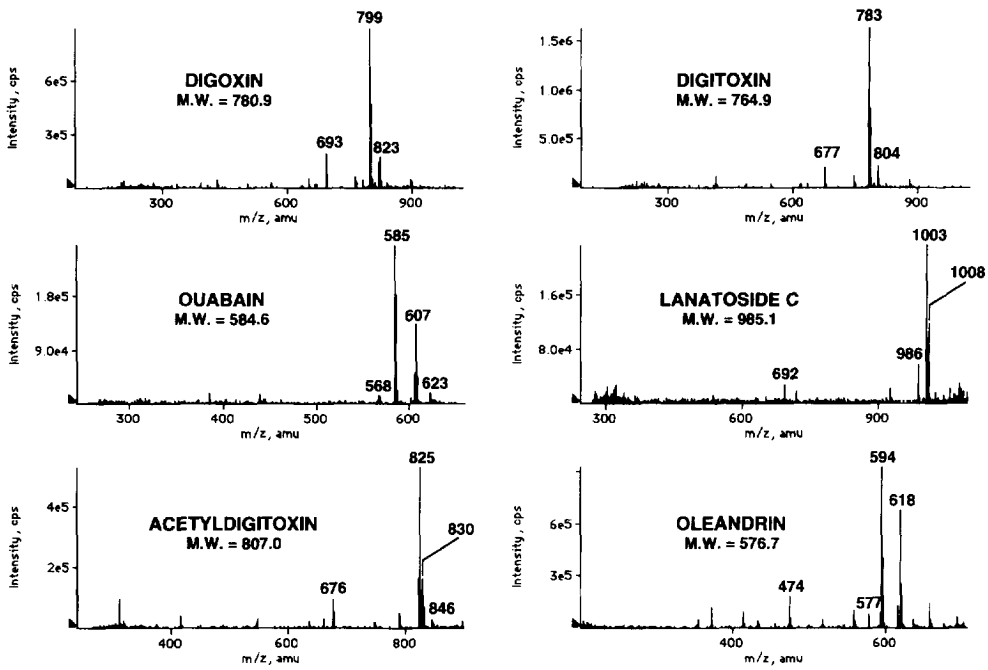

Fig. 1. Positive-ion, ISP mass spectra of digoxin, digitoxin, ouabain, lanatoside C, acetyldigitoxin, and oleandrin. Conditions: infusion (5 μl/min) of a 10.0 μg/ml solution of each drug in methanol–acetonitrile–2 m*M* NH_4COOH, pH 3.0 buffer (10:5:45, v/v).

predominate over the former mechanism: as a matter of fact, for all drugs tested but ouabain, the major ion recorded was not the protonated molecular ion $[M+H]^+$ as expected, but the related ammonium cluster $[M+NH_4]^+$. Minor formation of sodium $[M+Na]^+$ and potassium $[M+K]^+$ clusters was also observed, especially for ouabain and acetyldigitoxin. Contrary to other analytes [18–22], the NH_4^+ clusters built on CGs appear to be rather stable structures, since increasing the potential in the region of OR failed to result in significant declustering or fragmentation of any drug tested, even at high voltages (>150 V). These preliminary results prompted us to select the following representative ions for SIM of digoxin, digitoxin, ouabain, lanatoside C, acetyldigitoxin, and oleandrin: *m/z* 799, 783, 585, 1003, 825, and 594, respectively.

Table 1
Main representative HPLC–ISP-MS ions for six cardiac glycosides (ions are listed in decreasing order of abundance; OR=20 V)

	MW	Main ions *(m/z)*			
Digoxin	780.9	799[b]	823	693	804[c]
Digitoxin	764.9	783[b]	677	804	788[c]
Ouabain	584.6	585[a]	607[c]	568	623[d]
Lanatoside C	985.1	1003[b]	1008[c]	986[a]	692
Acetyldigitoxin	807.0	825[b]	830[c]	676	846[d]
Oleandrin	576.7	594[b]	618	474	577[a]

[a] $[M+H]^+$; [b] $[M+NH_4]^+$; [c] $[M+Na]^+$; [d] $[M+K]^+$.

Fig. 2 presents the chromatogram obtained by injecting onto the column 5 μl of a methanolic blend of the five analytes and the I.S. (each drug at 1.0 μg/ml, i.e. 2 ng injected) and collecting the data in SIM mode using the ions cited above, together with the different extracted chromatograms (XICs) got by reprocessing these data and focusing on each one of the ions tested, respectively. In this experiment as in subsequent ones, acetyldigitoxin was always found to elute in the form of two peaks of unequal abundance (average retention times: 8.66 and 9.01 min); the reason for this is that acetyldigitoxin from Sigma is a mixture of α- and β-isomers in a ratio ca. 2:1 (only the α-acetyldigitoxin being used in therapy). Owing to the constant disproportion between the two peaks monitored at *m/z* 825, we

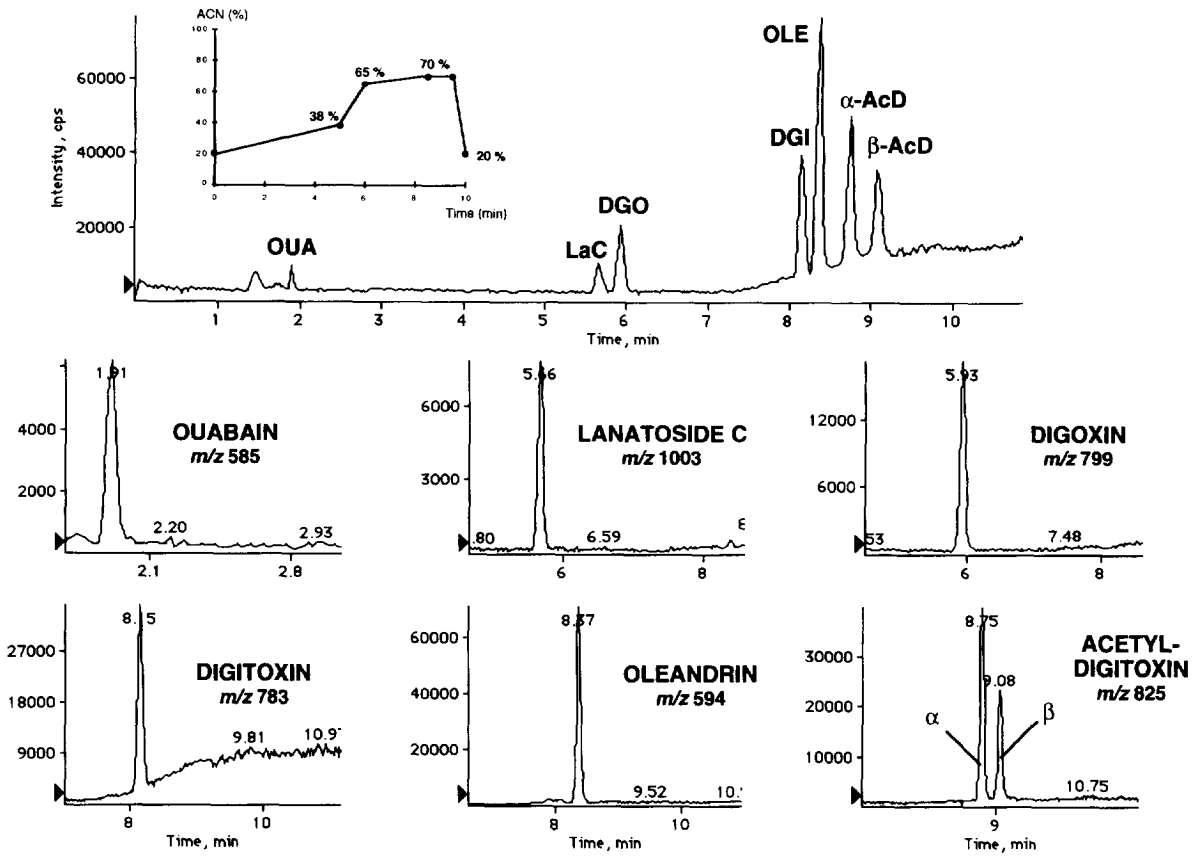

Fig. 2. Upper graph: chromatogram of a mixture of ouabain, lanatoside C, digoxin, digitoxin, oleandrin, and α/β-acetyldigitoxin (each drug at 1.0 μg/ml in methanol), recorded in SIM at *m/z* 585, 1003, 799, 783, 594, and 825 (column: 4 μm NovaPak C_{18} (Waters), 150×2.0 mm, I.D.); inset, gradient of acetonitrile–2 m*M* NH_4COOH, pH 3.0 buffer (flow-rate 200 μl/min, post-column split 1:3). Lower graphs: extracted chromatograms (XICs) on each of the representative *m/z* values.

assumed the former and the latter one to correspond in all likelihood to α- and β-acetyldigitoxin, respectively.

All our attempts to develop an isocratic separation remained unsuccessful, due to the large range of polarities exhibited by the different drugs tested. A 3-slope, S-shaped gradient (Fig. 2) was found necessary to conciliate a reasonable run time with an adequate resolution between ouabain (very polar), lanatoside C, digoxin (both of medium polarity), and the more apolar digitoxin, oleandrin, α-acetyldigitoxin and β-acetyldigitoxin. Variations of the solvent strength over a wide range were found to produce only slight variations in ouabain retention times: even at 10% acetonitrile, ouabain is almost not retained on the column ($k'<0.2$). An optimization of our method focused on ouabain determination would probably necessitate the choice of a less apolar stationary phase. The elution order of the other CGs (lanatoside C>digoxin>digitoxin>acetyldigitoxin) was in accordance with the preliminary results of Marquet et al. also obtained using a HPLC–ISP-MS coupling [23].

A single-step, liquid–liquid extraction using a mixture of chloroform–2-propanol (according to Kwong and McErlane [16]) was employed for reasons of speed and convenience. Absolute recovery was determined for the different CGs by extracting and assaying in the SIM mode samples of blank plasma spiked with ouabain, lanatoside C, digoxin, digitoxin, oleandrin, and acetyldigitoxin (each drug at 20 ng/ml), and by comparing the representative

peak heights of these extracted samples with those of unextracted methanolic standards at the same concentration. Results (mean±S.D. of six successive experiments) were: <10%, 71.0±7.6%, 66.7±9.3%, 90.2±5.1%, 86.8±8.3%, and 89.8±6.0% (sum of isomers), respectively. The recoveries found were good to excellent, except of course for ouabain (which was predictable owing to its very poor solubility in chloroform [24]). Considering the limited prescription of ouabain (used only intravenously in emergency situations) and the difficulties previously mentioned in developing a unitary chromatographic procedure for CGs comprising this drug, we decided to give up and exclude ouabain from our further experiments.

Oleandrin was chosen for I.S. since this toxic glycoside from *Nerium oleander* is closely related to other CGs, has no medical use in developed countries, is well co-extracted, and elutes with good resolution towards the analytes tested. Quantification was realized by computing peak height ratios (drug:I.S.) from XICs of the sample extracts analyzed in SIM mode, and comparing them with the calibration curves. These six-point standard curves were constructed for each CG by assaying blank plasma samples spiked to contain the drug at concentrations of 0, 1.0, 5.0, 20.0, 50.0, and 100.0 ng/ml (each concentration tested in duplicate); for acetyldigitoxin we assumed the given α:β ratio of 2:1 to be accurate, so that we considered the target concentration of α- and β-acetyldigitoxin to be respectively 2/3 and 1/3 of the added concentration of acetyldigitoxin at each datapoint tested. Results showed an excellent linearity for all analytes over the concentration range tested, with the following curve equations (y=height ratio drug:I.S.; x=drug concentration in ng/ml) and correlation coefficients (r): lanatoside C, $y=0.012x-0.016$ ($r=0.992$); digoxin, $y=0.023x-0.009$ ($r=0.997$); digitoxin, $y=0.029x+0.002$ ($r=0.992$); α-acetyldigitoxin, $y=0.067x-0.014$ ($r=0.999$); β-acetyldigitoxin, $y=0.049x-0.023$ ($r=0.995$).

Within-run accuracy and precision for the assay were determined by extracting and assaying aliquots of pure plasma fortified with lanatoside C, digoxin, digitoxin, and acetyldigitoxin (each drug at 20 ng/ml; six replicates). Results are given in Table 2. The day-to-day precisions, estimated by daily analysis of an aliquot of plasma loaded with the analytes at 20 ng/ml over a period of ten days, ranged from 12.7% (lanatoside C) to 18.9% (digoxin). This was found acceptable, however we recommend performing a new calibration (or at least a resloping with a high and a low calibrator) at the beginning of each chromatographic session. The within-run variability of retention parameters was studied by successively running ten plasma samples loaded with the CGs at 2.0, 5.0, 20.0, 50.0, and 100.0 ng/ml (i.e. two replicates/concentration). The repeatability of the chromatographic separation (Table 3) appears excellent, especially for the more apolar compounds. Combined with the specific mass detection, this allows the unequivocal identification of each of the analytes investigated, including in "general-unknown" screening situations. Even at the highest concentration tested, the overall resolution of the

Table 2
Accuracy and precision for the assay

	Concentration added (ng/ml)	Concentration found[a] (ng/ml)	Accuracy[b] (%)	Precision[c] (%)
Lanatoside C	20.00	19.45±1.88	2.8	9.7
Digoxin	20.00	20.88±2.71	4.4	13.0
Digitoxin	20.00	19.93±2.10	0.4	10.6
α-Acetyldigitoxin	13.33[d]	13.13±0.75	1.5	5.7
β-Acetyldigitoxin	6.67[d]	6.92±0.9	3.7	13.0

[a] Mean±S.D. from six replicates.
[b] (|Mean of found concentrations−added concentration|)/added concentration.
[c] S.D. of found concentrations/mean of found concentrations.
[d] Assuming the α:β ratio of isomers to be 2:1 as claimed by Sigma.

Table 3
Retention parameters for six cardiac glycosides

	Retention time (min)[a]	C.V. (%)	Relative retention time (min)[a,b]	C.V. (%)	k'	R_s^c
Lanatoside C	5.74±0.17	2.96	0.692±0.022	3.18	2.59	1.70
Digoxin	6.00±0.17	2.83	0.724±0.021	2.90	2.75	10.38
Digitoxin	8.08±0.03	0.37	0.974±0.002	0.21	4.05	1.55
Oleandrin (I.S.)	8.30±0.03	0.36	-	-	4.19	2.43
α-Acetyldigitoxin	8.66±0.04	0.46	1.044±0.001	0.10	4.41	2.23
β-Acetyldigitoxin	9.01±0.04	0.44	1.085±0.001	0.09	4.63	-

[a] Relative retention time vs. the I.S. oleandrin.
[b] Mean±S.D. from ten runs at five different concentrations between 2.0 and 100.0 ng/ml.
[c] Resolution vs. following peak, measured at 100 ng/ml: $R_s = 1.18\ (t_2 - t_1)/(w_2 - w_1)$, with t=retention times and w=peakwidths at half-height (from Ref. [25]).

method was found optimal, all R_s values being ≥1.5 according to Snyder et al. [25].

Our limits of detection (determined by assaying in SIM mode pure plasma samples spiked with decreasing amounts of the CGs until a response equivalent to two times the background noise was obtained for each analyte on the corresponding XIC) were as follows: lanatoside C, 0.60 ng/ml; digoxin, 0.25 ng/ml; digitoxin, 0.20 ng/ml; α-acetyldigitoxin and β-acetyldigitoxin, 0.15 ng/ml. These results are satisfactory for digitoxin and acetyldigitoxin (therapeutic concentrations in the range 5 to 30 ng/ml), and also for digoxin and lanatoside C (active in the range 0.5 to 2.5 ng/ml). Owing to its better sensitivity and widespread use, RIA however remains the technique of choice for therapeutic drug monitoring of digoxin; on the other hand, the high specificity of our technique makes it very convenient for the forensic investigation of poisoning cases where a result expressed as "digoxin equivalents by RIA" is not acceptable.

Alternatively this method may be useful for the absolute identification and quantification of oleandrin in suspected poisonings by *N. oleander*, using either digoxin or digitoxin as the internal standard. As an example, Fig. 3 shows the chromatogram of a plasma sample from a 24-year-old female who tried to commit suicide by ingesting a home-made "tea" prepared with leaves and branches of oleander; this observation dates back to June 1988 [26], but samples of biofluids from this patient had been preserved at −18°C in order to wait for an appropriate analytical procedure. Even in the SIM mode (m/z 594), the chromatogram appears complex, which likely results from: (1) the coelution of the numerous other plant compounds that have been reported to be present in oleander extracts [27]; and (2) the unavoidable degradation of the sample after a seven year storage; nevertheless an important peak was observed at the expected retention time of oleandrin (8.32 min), and the TIC (m/z 520 to 640) subsequently performed on the same sample allowed oleandrin to be formally identified on the basis of its typical ions at m/z 594, 618 and 577.

4. Conclusion

The present method is the first HPLC–MS procedure described for the analysis of digoxin and several other CGs in human plasma. Owing to the single-step liquid–liquid extraction and mass detection, it is simple, rapid, and highly specific. It may represent an interesting complement (rather than an alternative) to RIA in situations requiring the absolute identification of the toxic responsible for a digoxin-like symptomatology.

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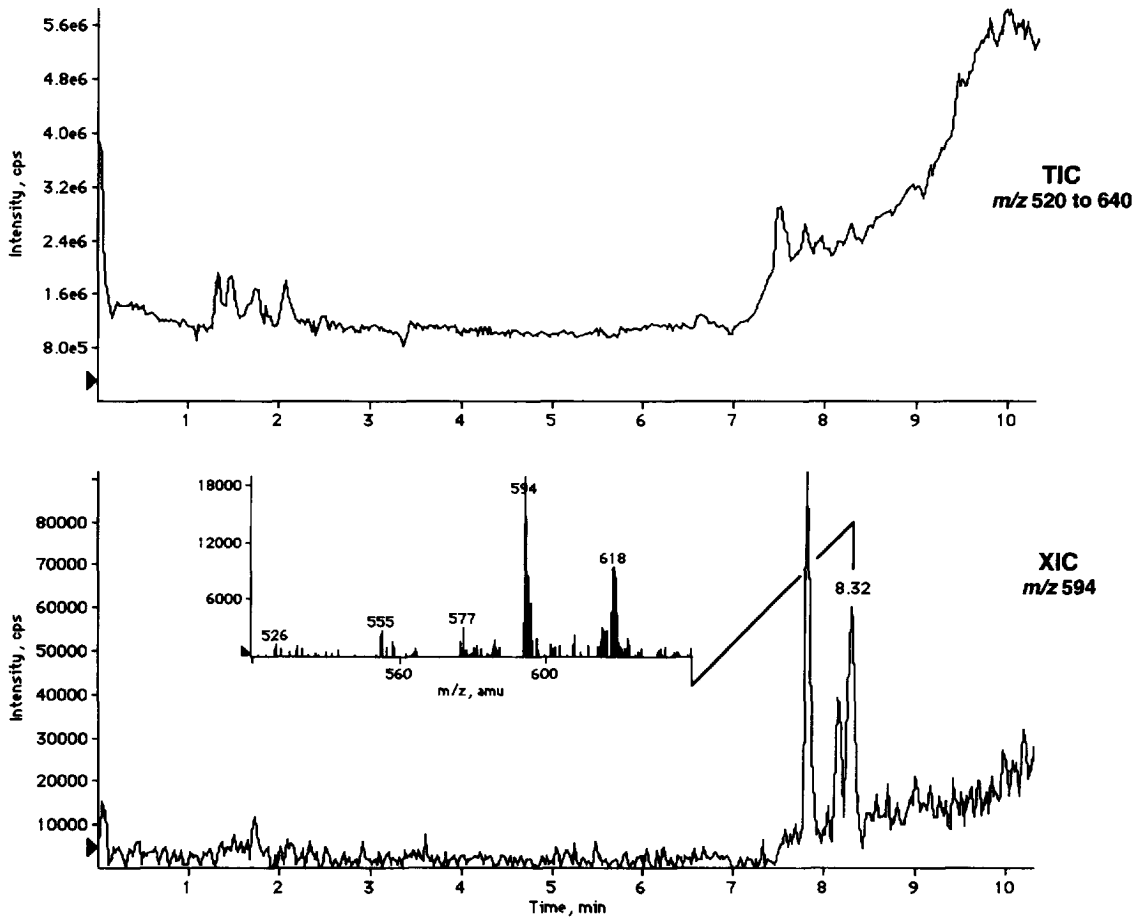

Fig. 3. Chromatogram from a plasma extract in a self-poisoning involving *Nerium oleander*. Upper graph: TIC recording (*m/z* 520–640). Lower graph: XIC at *m/z* 594; inset, ISP spectrum of the peak at 8.32 min. Chromatographic conditions as in Fig. 2.

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