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Note

High-performance liquid chromatographic method for isolation of tritiated digoxin and metabolites in urine

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High-performance liquid chromatography (HPLC) with UV detection has been used successfully to isolate and quantitate digoxin, digoxigenin and its mono- and bis-digitoxosides [1, 2], but not with the sensitivity necessary for assay of therapeutic concentrations in biological fluids. To achieve this level of sensitivity, it has been necessary to combine HPLC and radioimmunoassay [3, 4]. The use of tritiated digoxin as a tracer gives improved specificity and sensitivity compared with UV detection and permits isolation and quantitation of [^{3}H]digoxin-12α and its metabolites digoxigenin and the mono- and bis-digitoxosides, each in combination with their dihydro metabolite if present. We have also found three additional peaks which remain of unknown composition. Our knowledge of the biotransformation of digoxin in man remains incomplete or controversial and improved methods for its study are necessary.

EXPERIMENTAL

Materials

The HPLC system used was Constametric II (Laboratory Data Control Division of Milton Roy, Riviera Beach, FL, U.S.A.) with a Partisil 10 ODS 25 cm × 4.6 mm I.D. reversed-phase column and a 5 cm × 2.1 mm guard column packed with Co-Pell ODS (Whatman, Clifton, NJ, U.S.A.). The injection port was Model 7105 from Rheodyne (Berkeley, CA, U.S.A.). The flow-rate was 2.0 ml/min and elution was carried out initially with 35% methanol and then with 45% methanol at a pressure of 2000 p.s.i. (138 bar). The solvent change was

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made after the digoxigenin-mono-digitoxoside peak started to descend. The UV detector used to monitor peaks at 220 nm was a modified Beckman Model 25 spectrophotometer (Beckman, Palo Alto, CA, U.S.A.). The chart speed was 0.2 in./min and the span 0.25 A. Eluted fractions of 0.5 ml were collected with an LKB Produkter fraction collector Model 7000 (Bromma, Sweden). [^{3}H]Digoxin and metabolites in 0.5-ml fractions were counted in Riafluor (New England Nuclear, Boston, MA, U.S.A.) using a Beckman liquid scintillation spectrometer, Model LS-330, with a counting error of <2%.

Spectranalyzed methylene chloride and methanol were purchased from Fisher Scientific (Montreal, Canada) and the methylene chloride later double distilled. The water was purified by reverse osmosis and a Milli-Pore A filter system. The mixtures of methanol and water used for elution were degassed and filtered under vacuum before application.

[^{3}H]Digoxin-12α (Lot No. 690-186) with a specific activity of 16 mCi/mg was purchased from New England Nuclear. Digoxigenin-bis-digitoxoside, digoxigenin-mono-digitoxoside and digoxigenin were purchased from Boehringer Mannheim (G.F.R.) (Lots 61016, 7681 and 154182 respectively), and dihydrodigoxin was a gift from Burroughs Wellcome (Research Triangle Park, NC, U.S.A.). The above four compounds were tritiated by New England Nuclear by catalytic exchange. Specific activities were 1.16 mCi/mg for [^{3}H]-digoxigenin-bis-digitoxoside, 1.01 mCi/mg for [^{3}H]digoxigenin-mono-digitoxoside, 2.3 mCi/mg for [^{3}H]digoxigenin and 4.4 mCi/mg for dihydrodigoxin. Dihydrodigoxigenin and [^{3}H]dihydrodigoxigenin were prepared by hydrolysis of dihydrodigoxin and [^{3}H]dihydrodigoxin in acid. The purity of all compounds except for the dihydro derivatives, was achieved and verified before use by thin-layer chromatography and Sephadex LH-20 chromatography [5]. Thin-layer chromatography was used for the purification of the dihydro compounds [6]. Keto-digoxigenin and epidigoxigenin were also purchased from Boehringer.

Procedures

Urine voided by healthy volunteers was used immediately for studies which involved assessment of recovery. As part of a study of the influence of gastric acidity on the biotransformation of digoxin [7], six volunteers were given 150 μCi [^{3}H]digoxin-12α and 250 μg unlabelled digoxin (Lanoxin, Burroughs Wellcome) down a naso-gastric tube, firstly with stimulation of acid secretion by a pentagastrin infusion, and secondly without such stimulation. Urine was collected serially for assessment of endogenously formed metabolites and frozen at −25° C until assayed. Some results on the specimens collected 0—5 h after drug administration are reported; other results will be reported in detail elsewhere. All urine specimens were centrifuged and the supernatant extracted three times with methylene chloride. The volume of urine extracted was calculated to contain approximately 30,000 cpm (usually about 5 ml) and the volume of methylene chloride used was four times the urine volume. Aliquots of urine before and after extraction, as well as methylene chloride extracts were counted and recoveries calculated. [^{3}H]toluene was used as an internal standard to correct for quenching. Extracts were taken to dryness under vacuum, reconstituted in 100 μl of methanol and the entire volume injected

into the injection port of the chromatograph. Glassware was treated with Siliclad (Clay Adams, New York, NY, U.S.A.). The state of the column was checked daily before assays were performed by monitoring the UV peaks produced by application of a mixture of standards of digoxin, digoxigenin and its mono- and bis-digitoxosides in methanol.

These studies were approved by an ethics committee and volunteers gave informed consent.

RESULTS AND DISCUSSION

UV detection of digoxin and metabolites

Aliquots (5 μg each) of digoxigenin, its mono- and bis-digitoxosides and digoxin in combination in 20 μl methanol were applied to the column and peaks recorded with the UV detector. Mean retention times for 14 runs with standard deviations were 5.2 ± 0.2 min for digoxigenin, 9.5 ± 0.4 min for the mono-digitoxoside of digoxigenin, 16.7 ± 0.5 min for the bis-digitoxoside of digoxigenin and 21.9 ± 0.7 min for digoxin. Variations of up to 5 min occurred with different columns. Keto-digoxigenin and epi-digoxigenin had the same retention time as digoxigenin, based on UV detection.

When the four drugs were dissolved in methanol and 20 μl of the combination injected, the limit of sensitivity of this HPLC system using UV detection considering peak heights and 2:1 signal-to-noise ratio, was 278 ng for digoxin, 50 ng for digoxigenin, 132 ng for its bis-digitoxoside and 150 ng for the mono-digitoxoside. Digoxin, for a given weight applied, gave the least UV absorbance with a peak height about one sixth of that obtained for the same amount of digoxigenin. These limits in sensitivity preclude use of this method for detection of digoxin and its metabolites in urine when therapeutic doses are used. Furthermore, considerable UV absorbing material is extracted from urine with methylene chloride giving a large peak from 0—5 min which obscures two peaks found after administration of [^{3}H]digoxin-12α and overlaps with the digoxigenin peak (Fig. 1). It has therefore been necessary to use tritiated digoxin to obtain amplification and improved separation.

Detection using ^{3}H-labelled digoxin and metabolites

[^{3}H]Digoxin-12α, [^{3}H]digoxigenin and its ^{3}H-mono- and bis-digitoxosides were added to urine in amounts comparable to that found in urine during the 24 h after ingestion of 150 μCi [^{3}H]digoxin-12α. A 5-μg amount of each compound in the unlabelled form was also added to each specimen before extraction. The retention times for peaks determined by measurement of radioactivity were about 1 min later than peaks found by UV detection due to the tubing leading to the fraction collector. Fig. 2 illustrates the excellent separation of the four compounds with radioactivity falling to baseline between peaks.

Results of recovery studies for digoxin, digoxigenin and its mono- and bis-digitoxosides, performed on three occasions, are shown in Table I. The digoxin concentration was 0.4 pmole/ml (0.3 ng/ml). Relative recoveries of the individual compounds were within 1% of the relative percentages added. The absolute recoveries of radioactivity for all four compounds ranged from

TABLE I

TRIPLICATE RECOVERIES OF TRITIATED DIGOXIN AND METABOLITES ADDED TO URINE

Extraction from 5 ml urine.

	pmole/ml added to urine	Relative radioactivity added to urine (%)	Relative radioactivity recovered (%)			Absolute radioactivity recovered (%)			Mean recovery (pmole/ml)	Standard deviation (pmole/ml)
			Experiment No.							
			1	2	3	1	2	3		
Digoxin	0.368	43	43	42	43	71	73	69	0.261	0.008
Bis-digitoxoside	1.71	10	11	11	10	73	78	71	1.27	0.06
Mono-digitoxoside	6.76	29	29	29	29	72	78	73	5.03	0.22
Digoxigenin	2.56	18	17	18	18	68	73	70	1.80	0.06
Total radioactivity recovered (%)						71	76	72		

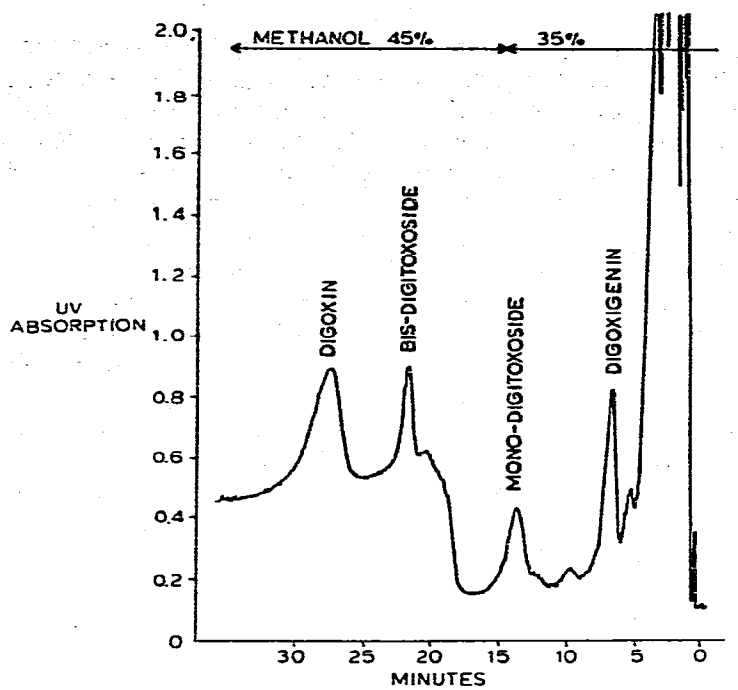

Fig. 1. UV tracing (220 nm) of eluate obtained after 5 μg each of digoxin, the mono- and bis-digitoxosides and digoxigenin, along with tritiated derivatives of each compound, were added to urine, extracted with methylene chloride, reconstituted in methanol and applied to a reversed-phase HPLC column. The solvent was changed from 35% to 45% methanol when the mono-digitoxoside UV peak started to descend. The high absorptivity recorded over the first 5 min is due to methylene chloride extractable material in urine unrelated to digoxin and metabolites.

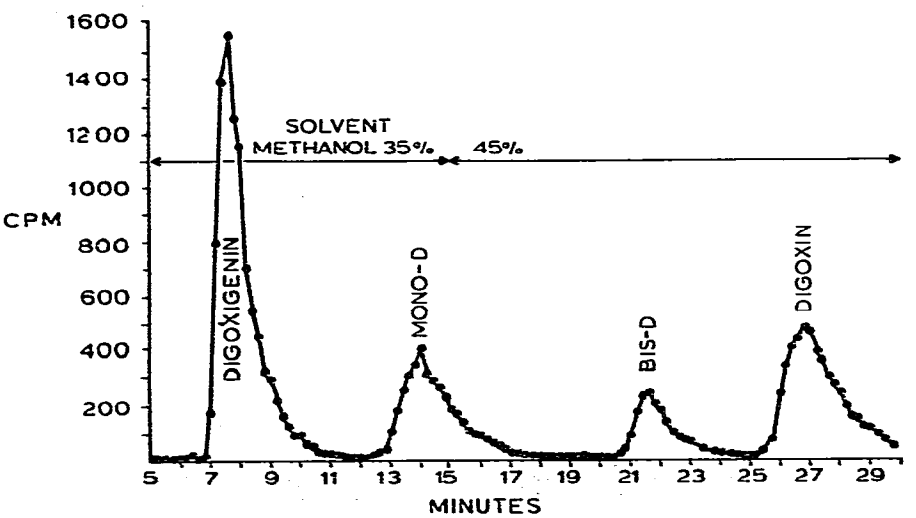

Fig. 2. Radioactivity profile in HPLC eluate obtained in the same study illustrated in Fig. 1 where tritiated and unlabelled digoxin, digoxigenin and its mono- and bis-digitoxosides were added to urine. The proportions of the four compounds found (digoxigenin 45%, mono-digitoxoside 18%, bis-digitoxoside 9% and digoxin 28%) were within 3% of those added to the urine.

68—78% (mean 73%), and differed by a maximum of 5% between the three runs for a given compound. The coefficients of variation for the four compounds ranged from 3—5%, considering the three recovery studies. There was no statistical difference between the recovery for digoxin and its three metabolites. Thus in the case of urine of unknown composition, when only a figure for recovery of total radioactivity can be determined, any error involved in making the assumption that recovery for all four compounds is the same will be small. Measured losses of radioactivity determined at each stage on three occasions averaged 3% during the extraction procedure, 8% on glassware (considering four transfers), 3% in the syringe, 3% in the injection port and on the column, and 8% in tubes used to collect fractions. Average total loss accounted for was 25%, compared with the average recovery of 73%.

Clear definition of peaks for urine extracts of the four tritiated compounds required maximum dpm of about twice the background of 50 dpm and a total dpm of 1000 when fractions were summed under peaks. These features provided sensitivity limits of about 40 pg for digoxin, 300 pg for digoxigenin and 650 pg for the mono- and bis-digitoxosides, considering a recovery of 70%. The limits of sensitivity for digoxin and metabolites in the studies involving urine collected after administration of [^{3}H]digoxin-12α, relate to the specific activity of [^{3}H]digoxin-12α of 16 mCi/mg and therefore were at the level of about 40 pg or less using 5 ml urine for extraction, or 8 pg/ml.

[^{3}H]Digoxin-12α and [^{3}H]digoxigenin-12α had the same retention times as their dihydro derivatives and it may be assumed that this also applies to dihydro metabolites of the mono- and bis-digitoxosides of digoxin. Dihydro metabolites were excreted in important amounts in some patients in two reports [8, 9] but only in small amounts in another [10].

Fig. 3 illustrates the radioactivity profile in the eluate obtained after application to the column of a methylene chloride extract of urine, reconstituted in

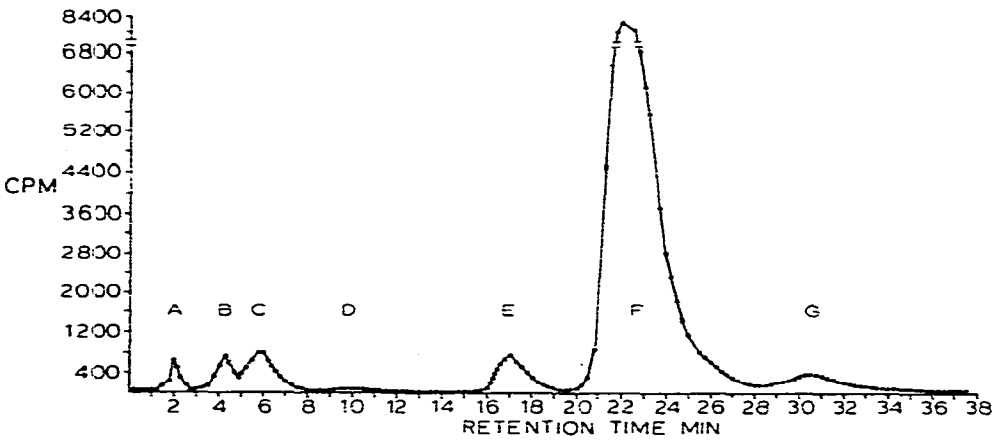

Fig. 3. Radioactivity profile obtained in reversed-phase HPLC column eluate after application of a reconstituted methylene chloride extract of urine collected from a volunteer 0—5 h after administration of 150 μCi [^{3}H]digoxin-12α. Most of the radioactivity (83%) is digoxin (peak F). The peaks C, D and E are digoxigenin and its mono- and bis-digitoxosides respectively. Peaks A, B and G are of unknown composition.

methanol. The urine was voided 0—5 h after administration of 150 μCi of [^{3}H]digoxin-12α without pentagastrin stimulation. The major peak, F, with 83% of the radioactivity is digoxin. Peaks C, D and E are digoxigenin and its mono- and bis-digitoxosides. The composition of peaks A, B and G is unknown. Peak A, at 2 min or 4 ml, starts to come off the column just after void volume: unstimulated it was 0—3% of radioactivity under the peaks, but averaged 5% after stimulation of gastric acid secretion with pentagastrin. Peak B, just preceding digoxigenin in the eluate, averaged 3% and 18% (maximum 31%) in the unstimulated and stimulated studies, respectively, and correlated with the amount of digoxigenin present. Peak G coming off the column after the digoxin peak, in contrast to peaks A and B, was present in greater amounts in the unstimulated series (mean 4%, maximum 9%) compared with the stimulated series (mean 1%) and correlated with the amount of digoxin present. The mean amounts of urine radioactivity extracted by methylene chloride were 46 and 85% for the stimulated and unstimulated series, respectively.

Although the primary objective of our method is the isolation and identification of metabolites of digoxin, it may also be used to quantitate digoxin, digoxigenin and its mono- and bis-digitoxosides, with the limitation that such values would include the dihydro metabolites if present. Quantitation of unlabelled digoxin and metabolites involves administration of labelled and unlabelled digoxin with known mass ratio and back calculation of mass in urine from the radioactivity under the HPLC peaks. The method assumes that the metabolism, excretion and recovery are similar for labelled and unlabelled digoxin.

Other methods of determining digoxin and metabolites such as radioimmunoassay and more recently HPLC with radioimmunoassay [3, 4] are methods for quantitation. Radioimmunoassay by itself, because of lack of specificity of antibody, may measure metabolites to varying degrees along with digoxin [11]. When HPLC is used to isolate digoxin and metabolites before use of radioimmunoassay as reported [4], only digoxin, digoxigenin and its mono- and bis-digitoxosides can be determined. Our method has detected three new extractable metabolites. The HPLC-radioimmunoassay method appears to have a lower limit of sensitivity of about 200 pg/ml for plasma, compared with about 8 pg/ml for urine with our method and also a lesser degree of precision.

In conclusion, an HPLC method has been developed using a reversed-phase column for isolation and quantitation of tritiated digoxin, digoxigenin and its mono- and bis-digitoxosides in urine; if one or more of the dihydro metabolites of these four compounds are present, then the combination is measured. Sensitivity at the 40-pg level or better was achieved for digoxin, digoxigenin and its mono- and bis-digitoxosides after the administration of [^{3}H]digoxin-12α. Recoveries averaged 73%. After administration of [^{3}H]digoxin-12α to volunteers, three additional radioactivity peaks were found in methylene choride extracts of urine. UV detection in the system used provided insufficient sensitivity and specificity to assay these compounds in the amounts found in urine after therapeutic doses.

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