Journal of Chromatography, 115 (1975) 625–628

CHROM. 8653

Note

Extraction of steroid diconjugates using Amberlite XAD-2 resin

MICHIO MATSUI, MISAKO HAKOZAKI and YUKO KINUYAMA
Kyoritsu College of Pharmacy, Shibakōen-1-Chome, Minato-ku, Tokyo 105 (Japan)
(Received July 22nd, 1975)

Amberlite XAD-2 resin has been successfully applied to the extraction of steroid conjugates from biological fluids such as urine[1] and bile[2]. Steroid conjugates are usually almost quantitatively adsorbed on the XAD-2 resin and subsequent washing with distilled water removes most of the solids present in the biological fluids. The conjugates on the resin can be quantitatively recovered in succession on elution with methanol.

During an investigation of the biliary metabolites of [4-^{14}C]testosterone (^{14}C-T) and [7-^{3}H]testosterone-17-sulphate (^{3}H-TS) in the rat[3] we observed that use of the XAD-2 resin as a means of separating steroid conjugates from rat bile resulted in relatively poor recoveries of the radioactivity in the methanol fraction, whereas the biliary metabolites of [1,2-$^{3}H_2$]testosterone-17-glucosiduronate (^{3}H-TGA) in the rat[4] usually gave more than 90% recoveries employing the same method. In the latter study ^{3}H-TGA was metabolized predominantly to $C_{19}O_2$ steroid monoglucosiduronates. In contrast, ^{3}H-TS was biotransformed mainly into $C_{19}O_2$ and polar hydroxylated steroid ($C_{19}O_3$, $C_{19}O_4$, etc.) diconjugates (mostly as disulphates). Simultaneously administered ^{14}C-T, which was metabolized to $C_{19}O_2$ and polar hydroxylated steroid conjugates, afforded relatively poor recoveries using the XAD-2 resin method. Thus, the increased hydrophilic nature of the ^{3}H-TS and ^{14}C-T metabolites due to hydroxylation of the steroid moiety and/or to diconjugation might reduce the affinity of these conjugates for the XAD-2 resin.

In the present study, the biliary metabolites of ^{3}H-TS and ^{14}C-T in the rat were obtained by Sephadex LH-20 column chromatography. These biliary metabolites were not adsorbed completely on the XAD-2 resin and modified procedures are described for their improved recovery from the resin.

EXPERIMENTAL

^{14}C-T (activity, 20.2 mCi/mmole), ^{3}H-TS (25 Ci/mmole) and ^{3}H-TGA (45 Ci/mmole) were purchased from New England Nuclear, Boston Mass., U.S.A. Details of the grade, source and preparation of reference steroids and reagents were reported previously[5]. Amberlite XAD-2 resin (Rohm & Haas, Philadelphia, Pa., U.S.A.; 100 g dry weight) was suspended in methanol and packed on to a column (700 × 20 mm I.D.) as a slurry. The resin was washed with 2 l of methanol and subsequently with

2 l of distilled water. Sephadex LH-20 (Pharmacia, Uppsala, Sweden) was processed as described by Laatikainen and Vihko[6].

A mixture of ^{14}C-T (0.41 μCi, 27 nmoles) and ^{3}H-TS (1.55 μCi, 27 nmoles) was administered intraperitoneally to male and female rats of the Wistar strain which weighed 250–300 g and had biliary fistulas. The biliary metabolites, ranging from 50 to 73% of the injected dose, were chromatographed on a column packed with Sephadex LH-20 and afforded monoglucosiduronate (^{3}H, 0.9–1.3%; ^{14}C, 22%), monosulphate (^{3}H, 6–11%; ^{14}C, 11–25%) and diconjugate (^{3}H, 20–47%; ^{14}C, 9%) fractions as described earlier[3]. The values in parentheses were calculated from the injected dose.

Each conjugate fraction was evaporated, dissolved in 10 ml of distilled water and passed through a column packed with Amberlite XAD-2 resin. The column was washed with 400, 200 or 100 ml of water, followed by elution with 400 ml of methanol. The diconjugates were solvolyzed in acidified ethyl acetate and the liberated steroids were examined by thin-layer chromatography (TLC) on plates coated with silica gel GF (E. Merck, Darmstadt, G.F.R.). The solvent was chloroform–acetone (29:1) and the procedure was as described previously[4]. Radioactive zones were detected with a Packard Model 7201 autoscanner, scraped and eluted with methanol. The radioactivity was counted with an Aloka LSC-502 liquid scintillation spectrometer in a toluene medium as reported previously[4]. Chloride was determined by the silver nitrate method[7]. Urea was determined by the diacetylmonoxime method[8].

RESULTS AND DISCUSSION

As a standard procedure for the separation of steroid conjugates, we employed the following condition: the column packed with Amberlite XAD-2 resin (100 g) was washed with 400 ml of distilled water following adsorption of the steroid conjugates and then eluted with 400 ml of methanol. Each conjugate fraction was processed by this procedure and typical recoveries of the radioactivity in both aqueous and methanol fractions are shown in Table I. Thus, it was apparent that the diconjugates were not adsorbed completely on the XAD-2 resin, in comparison with other conjugate fractions. In order to obtain information concerning the nature of the diconjugates,

TABLE I

PERCENT RECOVERY OF VARIOUS STEROID CONJUGATES FROM AMBERLITE XAD-2 RESIN

The column was washed with 400 ml of distilled water, followed by elution with 400 ml of methanol.

Conjugate fraction	*Rat*	*Radioactivity added (dpm)*		*Radioactivity recovered (%)*			
				Aqueous fraction		*Methanol fraction*	
		^{3}H	*^{14}C*	*^{3}H*	*^{14}C*	*^{3}H*	*^{14}C*
Monoglucosiduronate	Male	5110	11,000	0	2.9	95.0	96.5
	Female	3740	10,600	0	5.0	105	92.0
Monosulphate	Male	5970	5960	7.5	17.6	91.3	78.0
	Female	15,300	51,600	13.4	9.3	88.2	96.1
Diconjugate	Male	95,400	4640	32.0	40.9	64.5	63.6
	Female	101,000	9840	25.0	29.8	70.9	68.0

these aqueous and methanol fractions were solvolyzed, extracted with ethyl acetate and examined by TLC. No substantial differences were observed in their hydrolysis rates. In the male rat, *ca.* 95% of the hydrogen-3 and 74% of the carbon-14 in both fractions were extracted with ethyl acetate. The corresponding values for the female rat were *ca.* 73 and 64% respectively. TLC examination of the liberated steroids gave the following results. The aqueous fraction from the male rat demonstrated the presence of metabolites corresponding to polar hydroxylated steroids (^{3}H, 24%; ^{14}C, 68%) and 5α-androstane-3β,17β-diol (^{3}H, 63%; ^{14}C, 10%), whereas the methanol fraction gave polar hydroxylated steroids (^{3}H, 11%; ^{14}C, 66%) and 5α-androstane-3β,17β-diol (^{3}H, 80%; ^{14}C, 17%). TLC examination of the aqueous fraction from the female rat showed the presence of polar hydroxylated steroids (^{3}H, 54%; ^{14}C, 43%) and 5α- androstane-3α,17β-diol (^{3}H, 34%; ^{14}C, 36%), metabolites corresponding to polar hydroxylated steroids (^{3}H, 42%; ^{14}C, 41%) and 5α-androstane-3α,17β-diol (^{3}H, 45%; ^{14}C, 34%) being isolated from the methanol fraction. These results confirm the incomplete adsorption of $C_{19}O_2$ and polar hydroxylated steroid diconjugates on the XAD-2 resin.

In order to improve the recovery of the diconjugates, the XAD-2 resin was washed with 200 ml of distilled water, followed by elution with 400 ml of methanol. Typical recoveries are given in Table II. Thus, the diconjugates with relatively poor recoveries ranging from 64 to 71% with the standard procedure were recovered to the extent of 75–85% using this modified procedure. For comparison, ^{3}H-TS (20,200 dpm) and ^{3}H-TGA (22,000 dpm) were treated by this procedure and these monoconjugates provided 99% recoveries. Additional studies were then made with sodium chloride and urea in order to determine to what extent inorganic and organic compounds were eluted in the methanol fraction. Each 100 mg of sodium chloride and urea was treated as above. Determination of these compounds revealed that they were recovered quantitatively in the aqueous fraction. Thus, the reduction in volume of water from 400 to 200 ml should have little influence on the contaminations by inorganic and organic compounds. High recoveries of the diconjugates were obtained on washing the XAD-2 resin with 100 ml of distilled water, followed by elution with 400 ml of

TABLE II

PERCENT RECOVERY OF STEROID DICONJUGATES FROM AMBERLITE XAD-2 RESIN USING THE MODIFIED PROCEDURES

Procedure	*Rat*	*Radioactivity added (dpm)*		*Radioactivity recovered (%)*			
				Aqueous fraction		*Methanol fraction*	
		^3H	^{14}C	^3H	^{14}C	^3H	^{14}C
A*	Male	13,600	2090	19.0	18.0	80.5	82.0
	Female	16,100	2430	23.4	13.6	74.6	85.0
B**	Male	13,600	2090	6.6	2.9	91.2	84.6
	Female	16,500	2950	6.1	4.1	98.8	91.5

* The column was washed with 200 ml of distilled water, followed by elution with 400 ml of methanol.

** The column was washed with 100 ml of distilled water, followed by elution with 400 ml of methanol.

methanol (Table II). However, 4% of the sodium chloride and 23% of the urea appeared in the methanol fraction. These results demonstrate that the reduction in volume of water results in a convenient procedure for recovering the diconjugates from the XAD-2 resin, although some contamination by inorganic and organic compounds is inevitable. However, the modified procedure should be especially applicable to the extraction of steroid diconjugates from sodium chloride solutions following Sephadex LH-20[6] or DEAE-Sephadex[9] column chromatography of steroid conjugates, which employs 0.01 *M* sodium chloride as solvent or a 0–0.8 *M* sodium chloride gradient as the moving solvent phase.

REFERENCES

1 H. L. Bradlow, *Steroids*, 11 (1968) 265.
2 H. Jirku and M. Levitz, *J. Clin. Endocrinol.*, 29 (1969) 615.
3 M. Matsui, Y. Kinuyama and M. Hakozaki, *Steroids*, 25 (1975) 637.
4 M. Matsui, Y. Kinuyama and M. Hakozaki, *Steroids*, 24 (1974) 557.
5 M. Matsui, F. Abe, K. Kimura and M. Okada, *Chem. Pharm. Bull. (Tokyo)*, 20 (1972) 1913.
6 T. Laatikainen and R. Vihko, *Steroids*, 14 (1969) 119.
7 I. M. Kolthoff and E. B. Sandell, *Textbook of Quantitative Inorganic Analysis*, Macmillan, New York, 1936, p. 540.
8 A. A. Ormsby, *J. Biol. Chem.*, 146 (1942) 595.
9 R. Hobkirk, P. Musey and M. Nilsen, *Steroids*, 14 (1969) 191.